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Measles Virus - Immunoprecipitation and Monoclonal Antibody Production

by

© David John Paul Rafter

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF MASTER OF SCIENCE

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SUPERVISOR

T. Thorlakson

R. G. MacSyk

DATE September 28 1981

DEDICATION

This thesis is dedicated to Jennifer, who was a constant source of inspiration. I will always be grateful for her faith in me, and her love.

ABSTRACT

Antisera to the membrane (M) protein and to the nucleoprotein (NP) of measles virus, were prepared in rabbits. Each of the antisera was characterized by the immunoprecipitation technique and found to be monospecific. These monospecific antisera were used to determine, by indirect immunofluorescence, the cellular location of M-protein and NP during infection. The antiserum to M-protein stained the cytoplasm of Vero cells. The NP antiserum stained both the cytoplasm and nucleus.

The NP was found to undergo breakdown in infected cells. This breakdown could be inhibited by aprotinin, a protease inhibitor specific for trypsin and chymotrypsin. As no protease function has been identified for the proteins of measles virus, it is believed that the proteolysis is a cellular function. The significance of proteolytic breakdown in the assembly of measles virus is discussed.

Persons who receive inactivated measles vaccine and are subsequently exposed to wild type virus may experience atypical measles. It has been postulated that these individuals are susceptible to atypical measles because the inactivated vaccine fails to induce antibodies to the F-protein as detected by hemolysis-inhibition. Matched (acute and convalescent) serum samples were analyzed by the immunoprecipitation technique. Sera taken during the acute stage of the disease did not precipitate radiolabelled F-protein. Convalescent sera, which had been collected three weeks later, did precipitate the F-protein. The absence of antibodies to F confirms the earlier work obtained by hemolysis inhibition tests. These findings support the concept that atypical measles is the result of an absence of antibodies to F-protein.

Once it was recognized that atypical measles appeared following the use of inactivated vaccines, an attenuated virus vaccine was introduced for widespread immunization programs. This vaccine was also used in "catch up" programs to reimmunize individuals who had previously received "killed" vaccine. The value of this "catch up" program was analyzed using matched serum samples obtained from children previously immunized with inactivated virus vaccine, who volunteered for reimmunization with the attenuated vaccine. Since in no case was reimmunization found to result in conversion to the production of antibody to F-protein, the attempt to irradicate atypical measles by this procedure should be re-evaluated.

The monoclonal antibody technique was employed to obtain a series of monospecific antibodies to the measles virus proteins. In initial experiments 17 clones secreting antibody to the measles Lec strain were obtained. Of these, two clones have been established as stable cell lines. Both of these clones secrete antibody specific for the NP of the virus.

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ABBREVIATIONS

The units for mass, length, volume and time have been abbreviated according to standard procedure.

AMPS	-	ammonium persulfate
BIS	-	N, N^1-methylene-bis-acrylamide
BSA	-	bovine serum albumin
CF	-	complement fixing
CFA	-	complete Freunds adjuvant
CPE	-	cytopathic effect
CPM	-	counts per minute
CS	-	calf serum
CsCl	-	cesium chloride
ddH_2O	-	twice distilled H_2O
DMSO	-	dimethylsulfoxide
DTT	-	dithiothreitol
F	-	hemolysis protein
FITC	-	fluorescein isothiocyanate
HA	-	hemagglutinin protein
HAT	-	hypoxanthine, thymidine, aminopterin
HI	-	hemagglutination inhibition
HLI	-	hemolysis inhibition
IgG	-	immunoglobulin G
K	-	thousand
K.I.U.	-	Kallikrein Inactivating Units
L	-	large protein

mAmps	-	milliamperes
MEM	-	minimal essential medium
m.o.i.	-	multiplicity of infection
M-protein	-	membrane protein
NDV	-	Newcastle disease virus
NP	-	paramyxovirus nucleoprotein
P	-	polymerase protein
PAGE	-	polyacrylamide gel electrophoresis
PEG	-	polyethylene glycol
PPO	-	2-5 diphenyloxalozone
RIA	-	radioimmune assay
RIPA	-	radioimmunoprecipitation buffer
"-" RNA	-	negative stranded RNA
"+" RNA	-	plus stranded RNA
RPMI	-	Roswell Park Memorial Institute
SDS	-	sodium dodecylsulfate
SSPE	-	subacute sclerosing panencephalitis
SV-5	-	simian virus-5
TEMED	-	N, N, N^1, N^1-tetramethyldiamine
TLCK	-	N-α-p-tosyl-L-lysine chloromethyl ketone HCl
TPCK	-	L-I-tosylamide-2-phenylethylchloromethyl ketone
$\geq$	-	greater than or equal to

CHAPTER I

Literature Review

A) Clinical Measles

The clinical disease caused by measles virus has largely been eradicated in North America and Europe (Morgan and Rapp, 1977). However, the virus is still of clinical and scientific interest as it has clearly been shown to cause the debilitating disease subacute sclerosing panencephalitis (SSPE). Furthermore, measles virus is the most serious of the acute infectious diseases of African children, with a mortality rate in some locations approaching 20% (Morley et al, 1963). The severity of the disease has been attributed to the nutritional state of the children, and the age of onset of the disease (Morley, 1974). In North America, measles is commonly observed in children of school age, but in Africa the mean age of children contacting the disease is 17 months (Morley and MacWilliams, 1961). The children commonly experience severe bronchopneumonia, laryngitis, diarrhea and manifestations of malnourishment (Morley, 1962). This is markedly different from the clinical picture observed in North America, where the initial manifestations of the disease are a high fever with conjunctivitis and a moderately severe hacking cough. The typical rash appears 3-4 days later, first on the forehead, spreads downward over the face, neck, and trunk, and appears on the feet lastly. The lesions persist for three days and then disappear.

As there are no well planned vaccination programs in African countries and other third world nations, worldwide eradication of the disease as has occurred for smallpox is unlikely.

B) Pathogenesis of Normal Measles Virus Infection

The typical sequence in the pathogenesis of natural measles virus infection is summarized in Fig. 1 (Yamanouchi, 1980). Measles virus invades the host through the respiratory tract and the initial infection begins in the regional lymph nodes of the respiratory system (Kempe and Fuliginti, 1965). This leads to a primary viremia which infects lymphoid tissues and the spleen. Virus growth in the lymphoid tissue results in the formation of Warthin-Finkeldy type giant cells characteristic of measles virus infection, and may induce the marked destruction of both T and B cells (Wesley, Coovadia and Henderson, 1978).

The secondary viremia spreads virus to the epithelial tissues (White and Boyd, 1973). Burnett (1968) hypothesized that enanthema usually referred to as Koplik spots, and exanthema are produced as a result of T-cell mediated delayed type hypersensitivity to virus antigen in the oral mucous membrane and epidermal cells respectively. Virus antigen has been demonstrated in skin biopsies during measles exanthema (Olding-Stenkvist and Bjorvatan, 1976). Children with congenital immune dysfunction of T-cells do not develop the rash of measles (Chino et al, 1979). These observations are compatible with Burnett's hypothesis.

Virus produced in the epithelial cells of the lung is excreted by coughing and transmits the infection to other susceptible contacts.

C) Measles Immunology

The immune system plays an essential role in the recovery from viral illness. Observation of the clinical course of virus infections in patients with various types of immunodeficiency provides indirect evidence for the relative importance of humoral and cell-mediated

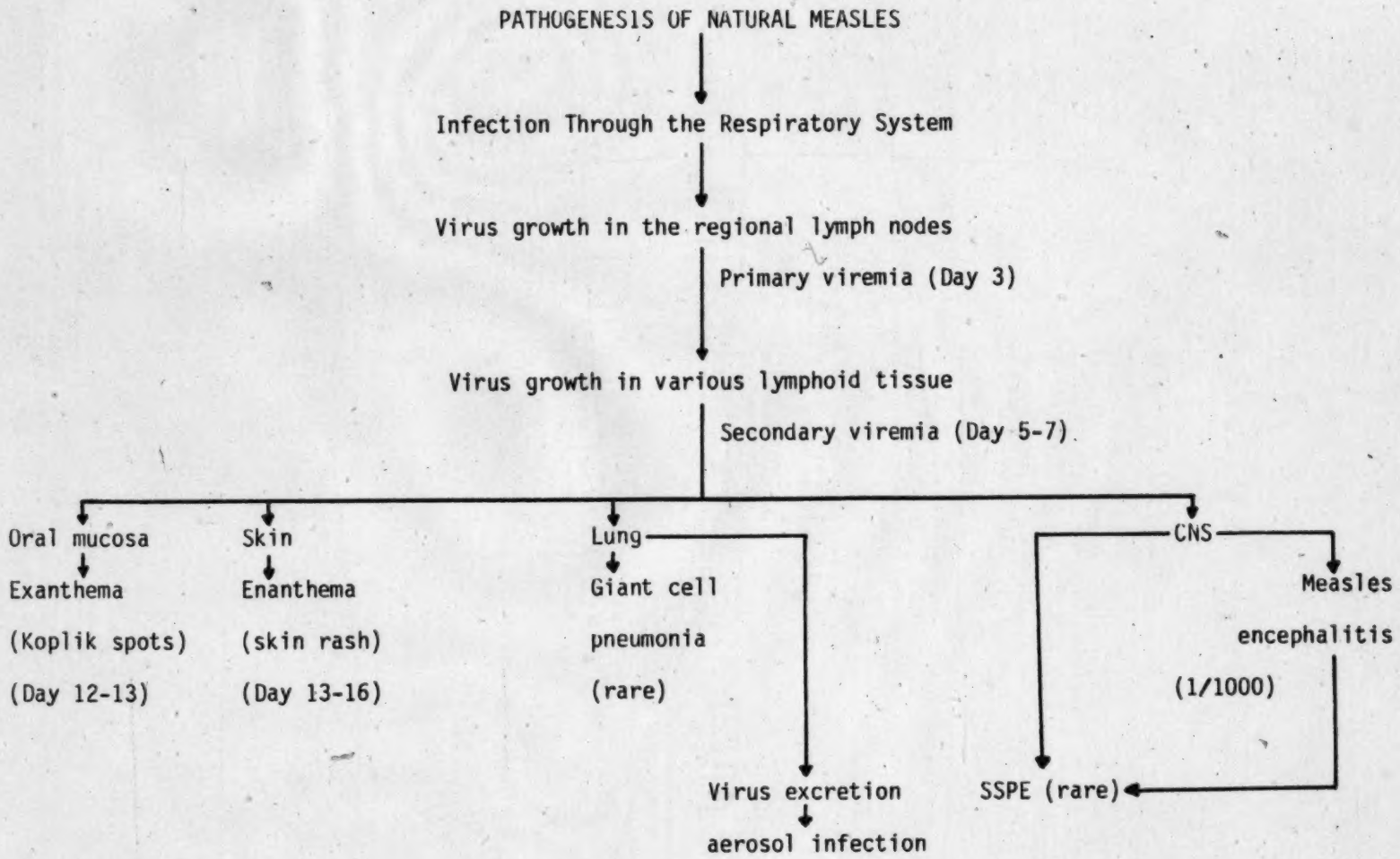

Fig. 1 Pathogenesis of natural measles virus infection (from Yamanouchi, 1980)

immunity in recovery from measles infection (Yamanouchi, 1980). Measles pneumonia (giant cell pneumonia) is frequently fatal in children whose cell-mediated immune functions are impaired, but have normal levels of immunoglobulin. Measles infection of children with X-linked agammaglobulinemia, who do not produce antibody but retain cell-mediated immune functions, follows a course indistinguishable from that in children whose immune systems are intact (Good and Zak, 1956). This implies that cell-mediated immunity is of special importance in recovery from measles.

A unique feature of measles virus infection is the marked immunosuppression occurring between two and five weeks following initial exposure in normal individuals (Yamanouchi, 1980). In 1908, von Pirguet recorded that patients with exanthema were unresponsive to the tuberculin skin test. Measles virus infections are characterized by a marked lymphopenia involving both T and B cells (Anderson et al, 1976). The decrease in T-cells seems to be related to the general suppression of cell-mediated immunity. McFarland (1974) attempted to elucidate the mechanism of measles-induced immunosuppression. In passive transfer experiments with mice, measles virus suppresses helper cell activity for T-cells. On the other hand, Valdimarsson and co-workers (1975) have reported that all T-cells, not just helper cells, are infected and no cell-mediated immune functions occur during infection. Therefore, the mechanism of immunosuppression observed by von Pirguet is still unclear.

D) Structure of Measles Virus

Measles virus is a member of the family Paramyxoviridae (Morgan and Rapp 1977). This family includes three geni: Paramyxovirus,

Morbillivirus and Pneumovirus (Table I). Measles virus, canine distemper virus, rinderpest virus, and pesti de petils ruminants virus comprise the genus Morbillivirus since they, unlike the other paramyxoviruses, do not possess a cell-associated neuraminidase activity (Choppin and Compans, 1975), nor do they absorb to neuraminic acid containing cellular receptors (Norrby, 1962). Measles virus is the only member of the morbilliviruses which agglutinates red blood cells (Howe and Schluederberg, 1970).

Measles virions, roughly spherical in shape, exhibit a great deal of pleomorphism, ranging in size from 120 to 270 nm in diameter. The bouyant density of intact virus particles as determined by bouyant density centrifugation is 1.23 to 1.25 g/cm^3 (Norrby et al, 1964).

The virus has an envelope which contains short projections or spikes on its outer surface (Fig. 2). Internally, the virus is largely comprised of a helical nucleocapsid. The inner surface of the viral envelope is lined by a membrane protein. The lipid of the enveloped viruses is derived from a host cell membrane (Choppin and Compans, 1975).

The genome of measles virus is a single stranded RNA of negative polarity, i.e. the genome cannot serve as message. Its molecular weight is 6.2×10^6 daltons, which is similar to the size of other paramyxovirus RNA genomes. The sedimentation co-efficient on sucrose gradients is 52S (Norrby et al, 1964). This is slightly larger than the 50S value for other paramyxoviruses (Kingsbury, 1972) and probably reflects differences in secondary structure.

PARAMYXOVIRIDAE

Family Paramyxoviridae

Genus: Paramyxovirus	Host
Species: Mumps	human
parainfluenza 1 (Sendai)	human, murine
parainfluenza 2	human, canine
parainfluenza 3	human, bovine
parainfluenza 4	human
Newcastle disease virus	avian
Genus: Morbillivirus	
Species: Measles virus	human
canine distemper virus	canine
rinderpest virus	bovine
pesti de petils ruminants virus	
Genus: Pneumovirus	
Species: Respiratory syncytial virus	human
respiratory syncytial virus	bovine
pneumonia virus of mice	

Table 1. From the study group of Paramyxoviridae, Vertebrate Virus Subcommittee, International Committee of Taxonomy of Viruses 1978

Measles virions are comprised of seven structural polypeptides (Tyrrell and Norrby, 1978, Table 2). The envelope contains two glycoproteins. The larger glycoprotein is responsible for hemagglutination (HA); (Breshkin et al, 1977). The smaller protein is the hemolysin or fusion (F) protein. The F-protein consists of two protein subunits, F_1 and F_2, linked by one or more disulfide bonds (Scheid and Choppin, 1977). The F_2 component is glycosylated (Tyrrell and Norrby, 1978). These two glycoproteins are believed to be linked via polypeptide tails extending through the envelope to the matrix or membrane (M) protein (Yoshida et al, 1976).

The nucleocapsid consists of three proteins and genomic "-" RNA. The most abundant protein of measles virus is the nucleoprotein (NP); it serves as the protective sheath for the genome (Mountcastle et al, 1970). The polymerase (P) and large (L) proteins are involved in genomic replication (Kolakofsky et al, 1974) and are associated with the nucleoprotein via non-covalent interactions (Markwell and Fox, 1980) (Fig. 2). Measles virions also contain a significant amount of cellular actin (Tyrrell and Norrby, 1978). The role of actin in the virus remains undetermined.

E) Measles Virus Infection and Assembly

Infection <u>in vitro</u> by measles virus is initiated by attachment via the HA glycoprotein to a receptor on the surface of the target cell (Trudgett et al, 1981). This is followed by fusion of the viral and cell membranes. The fusion is mediated by the F-protein (Hsu et al, 1979, Choppin and Scheid, 1980). Following fusion of the membranes, the nucleocapsid is released into the cytoplasm (Fraser and Martin, 1978).

Measles Virus Proteins

Protein	Molecular Weight[a]	Location[b]	Glycosylation[c]	Phosphorylation[d]
Large (L)	~200,000	internal	no	
Hemagglutinin (HA)	80,000	external	yes	no
Polymerase (P)	72,000	internal	no	yes
Nucleoprotein (NP)	60,000	internal	no	yes
Hemolysis[d] (F_0)	~ 60,000	external	yes	no
(F_1)	41,000	external	no	no
(F_2)	23,000	external	yes	no
Membrane (M)	36,000	internal	no	no
Actin	42,000		no	yes

Table 2: The proteins of measles virus

a + c) Tyrrell and Norrby, 1978

b) Hardwick and Bussell, 1978

d) Robbins and Bussell, 1979

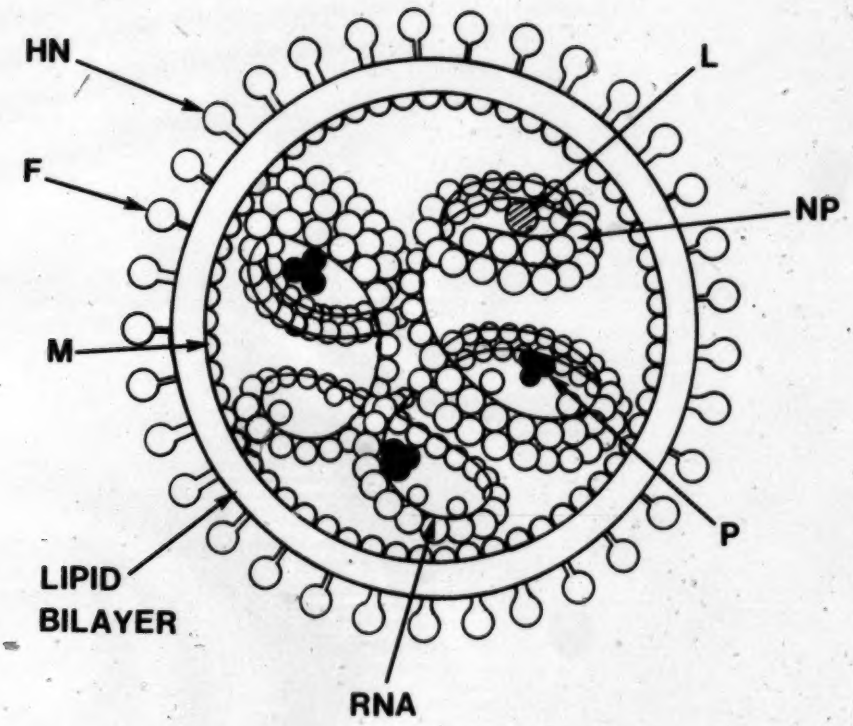

Fig. 2. Schematic representation of the structure of the paramyxoviruses. Taken from Markwell and Fox (1980).

The synthesis of viral mRNA is then initiated on the nucleocapsid template (Glazier et al, 1977, Seigfried et al, 1978). It is thought that the L-protein is responsible for this synthesis (Chinchar and Portner, 1981). Following the initiation of protein synthesis, RNA complementary to the genome (plus, "+" RNA) is synthesized. Both "-" RNA and "+" RNA are found in equimolar amounts in the infected cell, however only "-" RNA will be encapsidated into infectious virions (Kolakofsky, 1976).

The glycoproteins are synthesized first on the rough endoplasmic reticulum and then transferred to the smooth endoplasmic reticulum (Knipe et al, 1977a, b). The proteins in association with the smooth endoplasmic reticulum migrate to the cell surface and are inserted into the plasma membrane. The proteins diffuse freely over the surface of the infected cell, during which the F-protein is cleaved into its biologically active form by a cellular enzyme (Homma and Ouchi, 1973). The M-protein is synthesized in the cytoplasm and rapidly migrates to the cellular membrane. The glycoproteins are concentrated into "patches" by an interaction between the glycoprotein carboxyterminus and M-protein. These "patches" become sites of budding following the migration of the nucleocapsid to these areas (Yoshida et al, 1979).

The nucleocapsid containing negative stranded RNA may be transported to the budding sites by cellular actin (Tyrrell and Ehrnst, 1979). The M-protein is believed to play the crucial role in the budding of measles virus. It serves as the signal between the surface glycoprotein and the nucleocapsid to initiate budding.

F) Vaccination Against Measles

The first step in obtaining a measles virus vaccine was achieved following the successful isolation of infectious measles virus by Enders and Peebles (1954). Following this breakthrough, the virus was passaged a number of times in cell culture and adapted to grow in embryonated chicken eggs (Enders et al, 1960). The virus was inactivated with formalin or solubilized in Tween 80-ether and used in early vaccination programs in North America and Europe.

The original vaccine used in the 1960's was successful in reducing the incidence of clinical measles and its complications (Bass et al, 1976). However, there were problems associated with the use of this vaccine. The most serious complication was the development of atypical measles in individuals who had received the killed vaccine (Fulginiti et al, 1967). Atypical measles had not been recorded prior to the use of the inactivated vaccine. Atypical measles is a disease characterized by fever, cough and the appearance of a rash primarily on the extremities and lower trunk. The head, neck and upper trunk are frequently free of the rash. The rash is variable. It may be maculopapular like typical measles, but may resemble chickenpox or purpura. Patients with the disease frequently have pulmonary infiltrates which can persist for several months. The patients have a rapid rise in hemagglutination inhibition (HI) and complement fixation (CF) antibodies for measles virus. Epidemics of atypical measles have been recorded (Nader et al 1968). In the spring of 1979 an epidemic of measles, including many cases of atypical measles, occurred in Alberta.

Today the use of "killed" vaccines is no longer employed in North America (Morgan and Rapp, 1977). A "live" vaccine attenuated through

further passage in embryonated eggs and tissue culture is used. Successful immunization with live vaccine, particularly the Schwartz vaccine, produces long lasting immunity with few complications (Schwartz and Anderson, 1965; Krugman, 1977). Unfortunately there have been reports of SSPE in children whose only known exposure to measles virus was the "live" vaccine (Landrigan and Witte, 1973; Schneck et al, 1967).

There are three serologically defined antibodies observed following a measles infection or vaccination with "live" vaccine. These are the HI, CF and hemolysis inhibition (HLI) antibodies. The HI antibodies are direct to the HA protein, HLI antibodies to the F-protein and CF antibodies primarily to the nucleocapsid (Norrby and Gollmar, 1972, 1975). However in individuals receiving "killed" vaccine, HLI antibodies are not detectable (Norrby and Langercrantz, 1975). It is this failure which is believed to be responsible for the inadequate protection against measles following re-exposure to wild type measles virus. The resulting infection is manifest as atypical measles. The pathogenesis of the unusual rash and atypical pneumonia in atypical measles remains unknown.

The appearance of atypical measles resulted in the use of "live" vaccines. The use of the early "live" vaccines alone often induced a high fever and illness not unlike clinical measles (McCrumb et al, 1961). This next led to the vaccination program for measles which used the "killed" vaccine followed by "live" vaccine (Krugman et al, 1963). The "killed" vaccine was used prior to the "live" vaccine to attenuate the clinical response to the "live" vaccine. However, it is now clear that the use of "killed" vaccine prior to the administration of "live" vaccine is sufficient to "prime" the recipient for the possibility of

atypical measles. Health officials have also embarked on extensive vaccination programs of children who have received "killed" vaccine. To date there is no scientific evidence that revaccination with "live" vaccine will induce antibodies to the F-protein and prevent atypical measles. In fact, there is some epidemiological evidence to suggest that these revaccination programs have been ineffective (King-Collier, 1981).

G) Subacute Sclerosing Panencephalitis

Measles virus is the causative agent in SSPE (reviewed in ter Meulen et al, 1972; Thormar et al, 1975). SSPE is a slowly progressive disease of the CNS, which most often affects younger children. In more than half the recorded cases of SSPE the patients have experienced an uncomplicated case of measles at less than two years of age (Schneck et al, 1967; Jabbour et al, 1972). The onset of disease follows a lag period of five to seven years. The mechanism by which the virus persists in the CNS is not known.

Patients with SSPE have elevated serum and CSF antibody titers to measles virus (Brody et al, 1972). Cocultivation of brain material with primary cell lines susceptible to measles virus infection has lead to the successful recovery of virus in many cases (Chen et al, 1969; Horta-Barbosa et al, 1969a; Payne et al, 1969). However the virus isolated behaves differently than "normal" or wild type measles virus. The differences, summarized below, fall into two categories.

I) Differences in Growth Pattern

i) SSPE isolates grow slower and release less infectious virus (Thormar et al, 1978)

ii) Some SSPE isolates remain cell-associated and shed no infectious virus (Bernstein et al, 1974)

iii) Fewer budding particles are seen at the surface of infected cells (Thormar et al, 1978)

iv) The host range for some isolates is restricted in vitro (Hamilton et al, 1973)

II) Biochemical Differences

i) The genome of an SSPE isolate has been reported to contain 10% more genomic RNA (Hall and ter Meulen, 1976)

ii) Failure to release virus may be associated with alterations occurring in the glycoproteins (Breschkin et al, 1977)

iii) The molecular weight of the structural polypeptides are the same for SSPE virus and wild type, with the exception of the M-protein. An SSPE isolate has been reported to have decreased electrophoretic mobility corresponding to an increase in molecular weight of 2,000 daltons (Hall et al, 1978).

The M-protein of measles virus is believed to play a key role in the pathogenesis of SSPE. A number of laboratories have reported that the antibody response to M-protein is markedly reduced or non-existent in SSPE patients (Hall et al, 1979; Wechsler et al, 1979; Trudgett et al, 1980). Furthermore in a recent report by Hall and Choppin (1981),

the M-protein was not found in SSPE brain tissue, whereas all the other structural proteins of measles virus were demonstrated in brain tissue from SSPE patients. These findings have lead to the hypothesis (Hall et al, 1979; Hall and ter Meulin, 1978; Wechsler and Fields, 1978), that an aberration in the genome of measles virus results in the failure of the virus to follow a normal lytic infection. A persistent infection results with little or no virus shed from infected cells of the CNS. This may prevent a normal immune response to measles and allow the virus to persist.

As it is accepted that M-protein plays a critical role in the assembly of measles virus, it is attractive to accept the hypothesis that a defect in M-protein in SSPE virus results in viral persistence. However, the data presented in supporting the lack of antibody response to M-protein, and the failure to demonstrate M-protein in infected brain tissue was dependent upon the immunoprecipitation technique. Confirmation of these unusual findings by other techniques is mandatory before generally accepting that a defect in M-protein is the mechanism of pathogenesis of SSPE.

H) Aims of Project

The aims of this study were to develop techniques to study the biology of measles virus in cell cultures and in applied clinical problems. The immunoprecipitation technique was developed to characterize the specificity of monospecific antisera produced by a) immunization of animals with purified antigens or, b) by the monoclonal-hybridoma technique. With these antisera, I wanted to study the distribution of measles antigen in infected cells. The monoclonal antibodies would be

valuable experimental tools in the comparison of SSPE and wild strains of measles virus. It was also my intention to use the monoclonal antibodies to study SSPE biopsy material.

The immunoprecipitation technique could also be used to study the humoral immune response of children with typical and atypical measles. These studies should confirm the absence of an antibody response to the fusion protein of measles in children immunized with "killed" measles vaccine. Furthermore, I believed it would be possible to determine the value of the revaccination program for children originally immunized with "killed" measles. If revaccination with "live" measles vaccine is to be of any benefit, one would assume that antibody to the F-protein should be produced. This was evaluated in my study.

CHAPTER II

Materials and Methods

A) Cell Culture

CV-1 or VERO cells were used to grow measles virus in cell cultures. Both these cell lines are derived from the kidneys of Cercopithecus aethiops, an old world monkey. These cell lines are easily maintained in minimal essential medium (MEM) with Earle salts containing 100 I.U./mL of penicillin, 100 µg/mL of streptomycin, 30 mL/L 7.5% Na_2HCO_3, 29.2 µg/ml of glutamine and 5% calf serum, (CS). The monolayers were disrupted by washing twice with (Trysin 1:250 at 0.25%, 0.5 mM dextrose, 100 mM KCl, 100 mM NaCl, 50 mM $NaHCO_3$ and 1 mM EDTA) (ATV) and split in the ratio of 3:1.

B) Measles Virus

The LEC strain of measles virus was used in this study. This virus was isolated from a patient with SSPE by co-cultivation of brain tissue with human fetal kidney cells (Thormar et al, 1978). The virus has been used in a number of laboratories and has been reported to establish both persistent and lytic infections (Thormar et al, 1978). The LEC strain which I used was originally obtained from Dr. Erling Norrby of the Karolinska Institute in Stockholm Sweden.

C) Measles Virus Production and Purification

The production and purification of measles virus followed the method of Tyrrell and Norrby (1978). Confluent monolayers of healthy CV-1 Vero cells grown in 250 cm^2 glass culture (Blake) bottles were infected with LEC virus at a multiplicity of infection (m.o.i.) of 0.01

in 10 mL of MEM 1% CS. At two h post infection this medium was aspirated into 5% hypochloride bleach and 100 mL of MEM 5% CS was added. After 48 h approximately 10% of the cells showed the cytopathic effect (CPE) characteristic of measles virus infection. The medium from these cells was decanted into bleach and replaced with MEM 3% CS. At five to six days post infection the cell monolayer was completely destroyed. The media was removed and clarified by centrifugation at 4°C for 10 min at 1,000 x g in a Beckman J-21 preparative centrifuge. The supernatant was filtered through Whatman no. 1 filter paper and centrifuged at 28,000 x g for 1 h in a JA-14 rotor. The virus pellet was suspended in 6 ml phosphate buffered saline (PBS) pH 7.4, pooled, and sonicated for 10 sec at 100 watts using a Bronsonic sonicator to disrupt aggragated virus. The virus was placed on 40% to 60% (w/v) discontinuous sucrose gradients and centrifuged for 1 h at 60,000 x g in a Beckman L5-75 ultracentrifuge at 4°C. The virus band appeared at the 40% to 60% sucrose interface and was collected by puncturing the bottom of the tube. The virus band was dialyzed against 1 L of PBS, pH 7.4, for 12 h to remove the sucrose. The virus was further purified by centrifugation on a linear 15% - 40% (w/v) potassium tartrate gradient in PBS, pH 7.4 in an SW-40 rotor at 95,000 x g for 2 h. The visible band was collected and dialyzed against 1 L of PBS, pH 7.4, to remove potassium tartrate. Following dialysis the single virus band was harvested.

D) The Radioimmunoprecipitation Technique

The immunoprecipitation technique utilizes the ability of protein A derived from Staphylococcus aureus to bind to the Fc receptor of most immunoglobulins. As protein A binds to the Fc portion of IgG it is pos-

sible to form complexes of antigen, IgG and protein A are formed, without affecting the antibody binding sites. Protein A does not cause a detectable alteration in the structure of IgG upon binding to it. Protein A is able to bind to the Fc receptor of IgG in the presence of ionic and non-ionic detergents. In addition, protein A can be easily coupled to Sepharose CL-4B without loss of biological activity. It is these properties upon which the immunoprecipitation technique was established following the guidelines described by Lamb et al (1979). This technique utilizes the binding of radiolabelled solubilized antigens to antibody in a detergent solution. The antibody-antigen complex is then selectively removed from solution by protein A bound to Sepharose CL-4B beads.

The radioimmunoprecipitation technique was used to characterize a number of specific antisera for measles virus. These included monospecific rabbit antisera to the NP and M-proteins prepared by Tyrrell and Ehrnst (1979). The radioimmunoprecipitation technique was also applied to acute and convalescent serum samples from children who were diagnosed as having atypical measles, and to preimmunization and postimmunization sera from school children who voluntarily received "live" measles vaccine (attenuated), and had previously been immunized with the "killed" vaccine (inactivated).

Confluent monolayers of Vero cells in 25 cm^2 plastic flasks were infected with measles virus. When the infected cells exhibited greater than 70% CPE the media was decanted, replaced with methionine deficient MEM 2% CS and incubated for 1 to 2 h. After the incubation, the cells were washed 3x with sterile PBS, and 3 mLs of methionine deficient media containing 2% CS and 200 µCi/mL ^{35}S-methionine was added (specific

activity 1212.4 Ci/mmol). After a 2 h incubation the cells were washed with (3x) PBS and solubilized in 3 mL of cold radioimmune precipitation assay buffer [RIPA buffer (100 mM NaCl, 1% deoxycholate, 1% sodium dodecylsulfate, 1% Triton X-100, Tris-HCl, pH 7.4, 1 mM methionine, 1 mM phenylmethylsulfonylflouride and 1,000 K.I.U. of Aprotinin per mL)]. Following a 15 min incubation the solution was centrifuged for 30 min at 21,000 x g in an SW-60 rotor using a Beckman L5-75 preparative centrifuge. The supernatant containing the solubilized radiolabelled proteins was used immediately for immunoprecipitation or stored at -20°C.

The immunoprecipitation of serum samples was performed as follows; 2×10^6 CPM of the radiolabelled antigens in RIPA buffer was added to 20 µL of serum in 450 µL of RIPA and incubated for 2 h at 4°C in capped conical microfuge tubes (1.5 mL). During the incubation period the samples were vortexed briefly at 15 min intervals. Following this 20 µL of a 1:1 slurry (w/v) of Staphylococcal protein A covalently bound to Sepharose 4B in phosphate buffer (pH 7.2) was added to the mixture and incubated for 1 h and vortexed at 15 min intervals. After completion of the incubation period the complex was centrifuged at 12,860 x g for 2 min in an Eppendorf microfuge and washed with 1 mL of RIPA buffer. The washings were repeated four times. After the final wash was completed, 100 µL of sample buffer in preparation for SDS-PAGE was added, and the samples boiled for 3 min. In some experiments a protease inhibitor was added to the medium during the period of radiolabelling. The protease inhibitors used were 0.1 mM N-α-p-tosyl-L-lysine chloromethyl ketone HCl (TLCK), 0.1 mM-L-I-tosylamide-2-phenylethylchloromethyl ketone (TPCK) or 300 K.I.U./mL of Aprotinin.

E) Sodium Dodecyl Sulfate Polyacrylamide Gel Elelectrophoresis

Measles virus proteins were visualized on polyacrylamide gels using a modification of the method of Laemmli (1970). Slab gels were employed similar to those described by Studier (1973). The slab gels had an overall dimension of 19 cm x 15 cm x 0.2 cm. The spacer gel was 4 cm in length and consisted of 4.5% acrylamide and 0.12% N, N^1 - methylene Bis acrylamide (Bis) yielding a bisto acrylamide ratmlio of 1 to 37.5. The spacer gel was prepared using 2.25 mL of 40% (w/v) acrylamide, 1.20 mL of 2% (w/v) Bis, 2.5 mL of 1 M Tris-H_3PO_4, pH 6.8, 3.85 mL of ddH_2O, 0.2 mL of 10% (w/v) SDS, 0.1 mL of 10% (w/v) ammonium persulfate (AMPS) and 0.1 mL of 10% v/v N, N, N^1, N^1-tetramethyldiamine (TEMED) and an equal volume (10 mL) of 1.2% agarose. The separation gel was 15 cm in length and consisted of 10% acrylamide and 0.18% Bis which was prepared by using 10 mL of 40% acrylamide, 3.60 mL of Bis (Bis to acrylamide ratio of 1 to 56), 5.0 mL of 3M Tris-HCl, pH 8.8, 21 mL of ddH_2O, 0.40 mL of 10% SDS, 0.12 mL of AMPS and 0.12 mL of TEMED. The proteins were denatured by boiling for 3 min in sample buffer (0.0625 M Tris-H_3PO_4, pH 6.8, 2% SDS, 0.2% dithiorietol (DTT), 0.1% disodium EDTA and 10% glycerol). The samples were applied directly to the gel after boiling. The electrophoresis buffer consisted of 900 mL of ddH_2O, 100 mL of 1.92 M glycine - 0.25 M Tris and 0.1% SDS. The pH was not adjusted. The running buffer contained ~0.5 mL of a 10% (w/v) solution of bromophenol blue, to follow the electrophoretic front. The electrophoresis was run at a constant current of 10 mAmps. The electrophoresis was terminated when the migrating protein front was 1 cm from the bottom of the gel.

F) Autoradiography

The polyacrylamide gels were prepared for autoradiography by the method of Bonner and Laskey (1974). The spacer gel was removed and the separation gel placed in 150 mLs of DMSO for 2 h. Following this the gel was impregnated with 2,5-diphenyloxazole (PPO), using a 22.2% (w/v) PPO-DMSO solution. Following a three h incubation the PPO was precipitated by the addition of 1 L of H_2O. After two h the gel was dried under vaccum onto Whatman no. 1 filter paper. The radiolabelled proteins were visualized by exposing the dried gel to Kodak X-omat X-ray film for 48 h at -70°C. The films were developed in an automatic X-ray film processor.

G) Monoclonal Antibody Technique

The MOPC 315.43 mouse myeloma cell line used for the fusion was obtained from Dr. T. Mossmann of the Department of Immunology at the University of Alberta. This cell line was maintained in RPMI-1640, 50 μM-β-mercaptoethanol and 10% fetal calf serum (FCS). The cells were split every four days in a ratio of 10:1.

Mice (Balb/c) were immunized intraperitoneally (i.p) with 5 μg of measles virus which had been rapidly frozen and thawed (6x) using a dry ice-acetone bath. The virus was also sonicated 3x at 10 sec intervals using 250 watts. Twelve days after immunization the mice were given a booster of measles virus (5 μg). The spleen cells were collected 3 or 4 days after the animals received the booster. In some experiments mice were immunized by intramuscular injection with 5 μg of virus in an equal volume of Freunds complete adjuvant (CFA). These animals received a

booster by i.p. inoculation at 6 to 8 weeks with disrupted virus in the absence of Freunds adjuvant.

The procedure for the fusion of myeloma cells with immune spleen cells was provided by Dr. T. Mossmann. The immunized mice were sacrificed and the spleen aseptically removed. The spleen cells were teased apart with 23 guage needles in warm RPMI-1640 10% FCS medium and placed in 50 mL conical centrifuge tubes. After a 2 min period the cells were decanted into a second tube, leaving behind lumps of fibrous tissue. The spleen cells were counted and mixed in a ratio of 10:1 with myeloma cells in RPMI-1640 10% FCS and pelleted at 1,000 x g for 10 min. The cell pellets were resuspended in RPMI-1640 medium without serum and repelleted at 1,000 x g. To each cell pellet, 2 mLs of 40% (w/v) polyethylene glycol RPMI-1640 was slowly added over a 2 min period, with gentle tapping to partially dislodge pellet. After a further 2 min incubation, 20 mL of RPMI-1640 lacking serum was added dropwise over 3-5 min. The cells were repelleted and the supernatant removed. The pellet was gently dislodged by tapping and resuspended in 25 mL of RPMI-1640 20% FCS and incubated for 1-3 h in a tissue culture dish at 37°C in 5% CO_2. The cells were not completely resuspended, with large lumps of cells remaining.

Following this incubation the cells were placed in selection media [50 μM-β-mercaptoethanol, 100 I.U. mL of penicillin, 100 μg/mL streptomycin, 20 I.U./mL of nystatin, 1×10^7 mouse red blood cells/mL, 1 mM ouabain, and HAT (100 μM hypoxanthine, 30 μM thymidine and 0.5 μM aminopterin)] at a final concentration of 1×10^5 myeloma cells per mL.

The cells were left undisturbed for 10-14 days, at which time the surviving cells, as clones, were macroscopically visible. By this time

there were sufficient numbers of cells to screen for antibody secretion to the medium.

H) Radioimmune Assay

An indirect radioimmune assay (RIA) was developed following the guidelines of Amesse and Payne (1980). The RIA is considered to be sensitive for antibody detection.

Whole measles virus in PBS, pH 7.4, was diluted to a concentration of 50 µg/mL and 100 µL was placed in the wells of 96 well polystyrol microtiter plates. The virus was allowed to dry in the wells over a 24 h period. Plates prepared in this manner were either used immediately or stored at -20°C. The antigen-coated wells were fixed by a 1:1 (v/v) solution of cold acetone and methanol for 10 min. The plates were washed 3x with PBS. To each well 25 µL of PBS containing 0.5% BSA was added to block nonspecific binding, and 75 µLs of the media from the wells with visible colonies was added. Each sample was tested in triplicate. Serum controls (20 µLs) were added to 100 µL of PBS-BSA in each well. The serum samples were tested at eight serial five-fold dilutions.

The plates were incubated for 1 h at 37°C in a moisture chamber. Following this incubation the plates were washed 10x with PBS, pH 7.4. 100 µL of radio-iodinated goat anti-mouse IgG (50,000 CPM) in PBS-0.5% BSA was added to each well and the plates were incubated for 1 h at 37°C in a moisture chamber. After this incubation the plates were washed with PBS pH 7.4 (10x). Each well was cut and placed in liquid scintillation vials containing 5 mL of Aquasol or Scintiverse and the total counts per minute (CPM) determined in a programmable Rack Beta (LKB)

scintillation counter. When available, a gamma counter was used to count the radioactivity in each well. This was easier since there was no need for scintillation fluid in such samples. Clones secreting antibody for measles virus proteins were identified when the CPM exceeded the negative controls by two-fold.

These clones were removed from the wells by aspiration into sterile pasteur pipettes and replated into new wells in fresh RPMI-1640 medium containing hypoxanthine and thymidine. The cells were then confirmed as producing measles specific antibody by rescreening using an indirect RIA. Attmepts were made to establish stable cell lines from each positive clone for preservation and/or growth of ascites tumors in mice. In addition a small number of cells were grown in media containing FCS which had the IgG removed by ammonium sulfate precipitation. The media from these cells were used to screen for the specificity of the antibody produced by radioimmunoprecipitation and autoradiography.

I) Iodination of Goat Immunoglobulin G by the Lactoperoxidase Method

For the RIA radio-iodinated goat anti-mouse IgG was prepared as described below (Bayse and Morrison, 1971). A 46 µL solution (0.15 mg. protein) of IgG was incubated at 37°C with 2 µL of freshly prepared lactoperoxidase (550 µg/mL) in PBS, pH 7.0, 1 µL of a 1/3300 dilution of H_2O_2 and 1 µCi of $Na^{125}I$. After 10 min an additional 2 µL of lactoperoxidase and 1 µL of H_2O_2 were added and the incubation continued. After an additional 10 min, 1 µL of H_2O_2 was added and the incubation continued for a total time of 30 min. This reaction is summarized below.

$$I_2 + H_2O_2 + 2Tyr \rightarrow 2\ MIT + 2\ H_2O,$$

$$I^- + H_2O_2 + Tyr \rightarrow MIT + H_2O + OH^-.$$

The reaction was terminated by cooling to 0°C for 20 min and the uncomplexed $^{125}I^-$ was inactivated by forming a salt with Na_2SO_3 (10 µL of 80 mM solution). The iodinated protein was separated from the free iodine on a 5 mL column of Sephadex G-25. Fifteen fractions of 0.5 mL were collected and 5 µL samples were counted to determine the peaks of radioactivity. Trichloroacetic acid (250 µL) was added to 25 µL samples of each fraction to precipitate protein. Counts were repeated on the supernatants to confirm that the radiolabel was protein bound. Only those fractions in which greater than 90% of the counts were protein bound were used in the RIA.

J) Immunofluorescence

Immunofluoresence was as described by Tyrrell and Ehrnst (1979). Vero cells were grown on glass slides (19 cm^2) in Leighton tubes and infected with measles virus at a m.o.i. > 1. At 48 h postinfection the glass slides were removed, washed 3x in PBS, pH 7.2, and placed in cold acetone for 20 min. The cells were washed 3x in PBS at 10 min intervals and allowed to air dry. Ten fold serial dilutions of each sera (0.2 mL) was added to a slide. The slides were incubated at 37°C for 1 h in a moisture chamber and then washed 3x with PBS, pH 7.2, at 10 min intervals. Sheep anti-rabbit fluorescein isothiocyanate (FITC) conjugate was diluted 1:10 with PBS, pH 7.2, and 0.1 mL applied to the slides. After an incubation for 1 h at 37°C in a moisture chamber the slides were washed 3x in PBS, pH 7.2, and the cells evaluated for positive fluorescence in a LEITZ microscope. The controls used were uninfected cells and infected cells examined with non-immune serums. Activation filters

for the FITC-staining were BG38, KP490, and LP455. The beam splitter was FT510 and barrier filters were 51-58.

K) Ammonium Sulfate Precipitation of Immunogloblin G From Fetal Calf Serum

It was found that FCS obtained from Flow Laboratories Ltd. (lot. nos. 29101464 and 12972452), contained antibodies which precipitated measles virus nucleocapsid proteins. This interfered with the interpretation of the immunoprecipitation results from positive antibody-producing clones. In order to eliminate this problem it was necessary to remove IgG from the serum, yet retain the properties of fetal calf serum which permit the growth of cells in culture.

A saturated solution of ammonium sulfate was prepared by the addition of 1000 gm to 1000 mL of ddH_2O and allowed to remain at room temperature for 24 h and the pH was adjusted to pH 7.2 by the addition of NaOH. FCS (50 mL) was brought to room temperature and 25 mL of the saturated $(NH_4)_2SO_4$ solution added dropwise with continuous stirring. It was important to add the ammonium sulfate solution slowly in order to prevent nonspecific precipitation of serum proteins. The precipitate of IgG was not visualized until 24.5 mLs of solution was added. Following completion of the addition of ammonium sulfate the mixture was allowed to stir gently for 1 h. The mixture was pelleted in a clinical centrifuge for 20 min at 1,500 x g and the supernatant decanted. This centrifugation was repeated once. The serum solution was dialyzed against 40 L of PBS, pH 7.4, at 4°C to remove the $(NH_4)_2SO_4$ (dialysis tubing had a MW cutoff of 1 K). Each dialysis volume was 4 L and 10 changes of buffer were performed. The presence of ammonium sulfate in PBS was

easily monitored as it forms a white precipitate with barium chloride at a pH $\geq$ 7.0. No $(NH_4)_2SO_4$ was detectable after the seventh change of dialysate. The serum was passed twice through a millipore filter with a pore size of 0.22 µm.

L) Growth of Hybridomas in Mice as Ascites Tumors

Growth of ascites tumors was as described by Kennett et al, 1980. Briefly, mice were injected intraperitoneally with 0.5 mL of pristane and then rested for 6 wk. The animals were then sublethally irradiated (450 rads using ^{137}Cesium as the source in a Gamma cell 40 chamber) and 10^7 hybridoma cells/mouse were injected. Two to four weeks later the ascites fluid containing monospecific antibody was collected by paracentesis.

M) Protein Assay

Total protein determinations for measles virus and ascites fluid were carried out using the Bio-Rad Protein Assay System.

N) Materials

The CV-1 cell line (ATCC CCL70), Vero cell line (ATCC CCL81), MEM, FCS, CS and RPMI-1640 were purchased from Flow Laboratories Inc. Staphylococcus Protein A-Sepharose CL-4B and Sephadex G-25 were purchased from Pharmacia Fine Chemicals. TLCK, TPCK, aminopterin, hypoxanthine, thymidine, BSA and Lactoperoxidase were purchased from the Sigma Chemical Company. Aquasol and ^{35}S-methionine were purchased from New England Nuclear, and Na^{125}I was purchased from the Edmonton Radiopharmaceutical Center. All reagents for the PAGE analysis were

electrophoretic grade purchased from BIO-RAD Laboratories. Freunds complete adjuvant and trypsin were purchased from Difco. PEG was purchased from the J.T. Baker Chemical Co. Goat antimouse IgG was purchased from Cappell Laboratories. Sheep anti-rabbit-FITC was purchased from Burroughs-Wellcome. Aprotinin was purchased from Boehringer Mannheim Ltd. The 25 cm^2 plastic tissue culture flasks were purchased from the Corning Co. All other chemicals and reagents were of the highest quality available and were purchased from either Fisher Scientific or CANLAB.

CHAPTER III

Characterization of the Antisera for the Nucleocapsid and Membrane Proteins of Measles Virus

Introduction

Monospecific antisera to the measles virus proteins NP and M were prepared by Tyrrell and Ehrnst (1979) to be used in the study of the assembly of measles virus. The antisera to the NP and M-proteins were prepared by immunizing rabbits with proteins excised from SDS-polyacrylamide gels. This methodology was performed since attempts to purify NP free from M-protein by other techniques had failed to yield pure protein. Antisera to the M-protein were prepared from rabbits immunized with M-protein cut from SDS-polyacrylamide gels or M-protein isolated by detergent extraction and separation on CsCl gradients (Norrby and Hammerskjold, 1972).

Serological analysis of the rabbit antisera did not demonstrate HI antibodies or HLI antibodies. The antisera exhibited complement fixing activity to an antigen preparation containing both NP and M. However this did not specify the monospecific nature of these antisera. The immunoprecipitation and autoradiography techniques were applied to these sera to determine their monospecificity. This work has been published (Tyrrell et al, 1980).

Results

The immunoprecipitation of measles virus proteins from the infected cell lysate is shown in Fig. 3. The serum from a patient who recently suffered from atypical measles was used to immunoprecipitate most of the

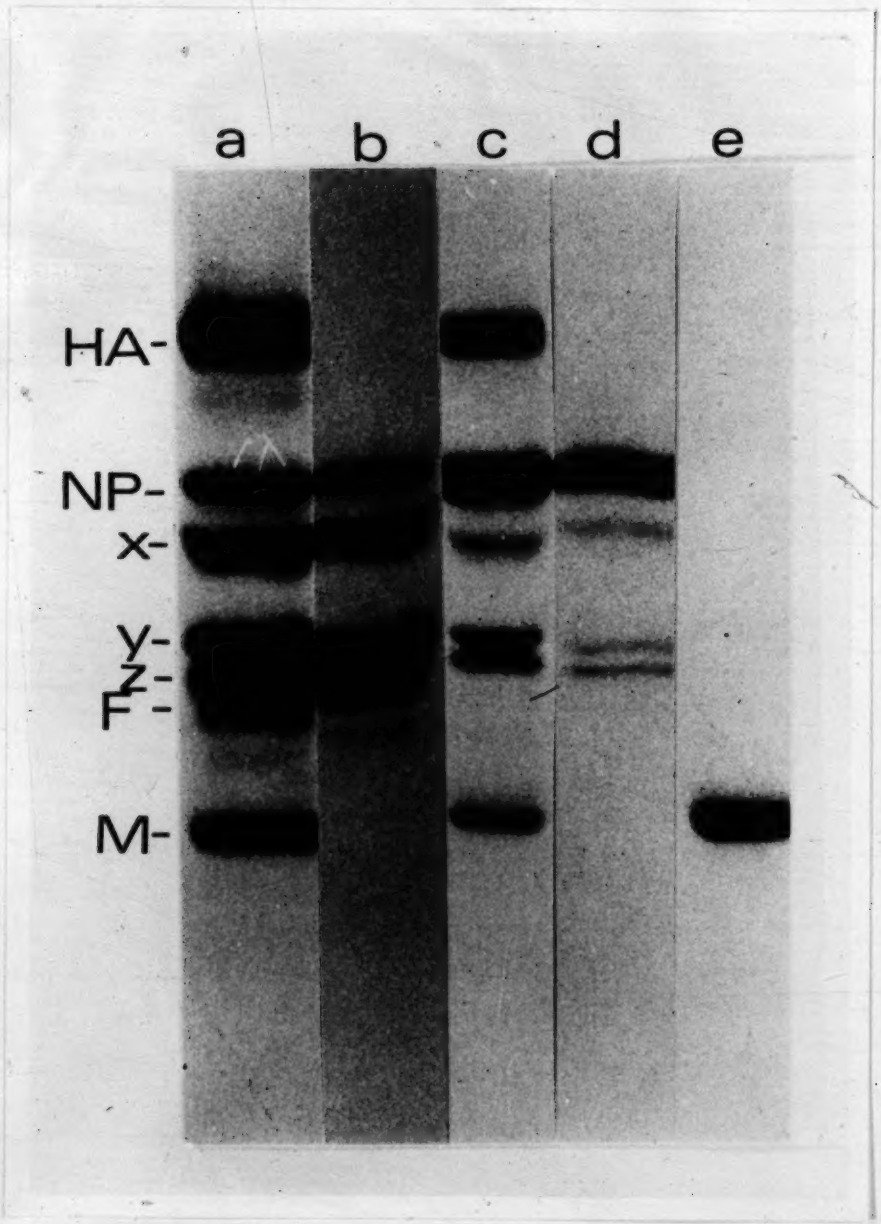

Fig. 3. Autoradiogram of the immunoprecipitated measles virus proteins. The viral proteins obtained from a hyperimmune serum in the absence (a) and presence (c) of aprotinin is depicted. In b and d, the precipitation of measles virus proteins by the antisera to NP in the absence and presence of aprotinin is depicted. In e the precipitation of M-protein by the rabbit antisera to M-protein is shown.

measles virus proteins (Fig. 3a). The specificity of the antisera for NP and M were examined by the immunoprecipitation technique and the results shown in Fig. 3. The antisera raised to the M-protein precipitated a single protein corresponding to M-protein of measles (Fig. 3e). The NP antiserum precipitated NP but in addition precipitated three proteins designated X, Y and Z (Fig. 3b). These proteins were also precipitated by the hyperimmune serum of atypical measles. The precipitation of three additional proteins in addition to the 60,000 daltons NP protein implied one of the following: a) the antisera prepared from SDS-polyacrylamide gels was not specific, b) NP was breaking down into smaller units either spontaneously or via cellular proteases or, c) the detergent solubilization of the infected cells in some way caused NP to break down into discrete subunits.

Of these explanations it was thought that detergent breakdown of NP was unlikely as solubilization of whole virus does not yield any proteins corresponding to X, Y and Z (Wechsler and Fields, 1978a). Furthermore, reports in the literature have shown that NP is extremely stable over a wide range of salt and ionic conditions (Heggeness et al, 1980; Compans and Choppin, 1968; Kingsbury et al, 1970). Thus it was felt that either the antiserum was not monospecific or protease digestion of NP was causing the appearance of X, Y and Z which would be precipitated by the NP antiserum. To test this hypothesis virus infected cells were treated with protease inhibitors at the time of labelling with ^{35}S-methionine. The protease inhibitors used were the serine protease inhibitors TPCK, TLCK, or Aprotinin, a protease inhibitor used clinically in the treatment of acute pancreatitis. It was found that a 0.1 mM concentration of TPCK or TLCK inhibited the incorporation of ^{35}S-

methionine into measles virus polypeptides. Aprotinin, on the other hand, at 300 K.I.U. per ml did not affect the incorporation of radiolabelled methionine into proteins.

Immunoprecipitation of Aprotinin treated measles infected cells which were labelled with ^{35}S-methionine is shown in Fig. 3c and d. With both the NP and hyperimmune antisera there was a dramatic decrease in amount of proteins labelled X, Y and Z. The incorporation of label into 60 K NP was subjectively increased. It should also be noted that these antisera failed to immunoprecipitate proteins from radiolabelled uninfected cells. Thus it appears, on the basis of this data, that the antisera raised to the NP and M proteins was monospecific.

Controversy exists in the literature as to the location of the NP and M proteins in infected cells. Some authors believe that both NP and M are found within nuclear inclusions (Miller, 1980; Wechsler and Fields, 1978a), while others suggest that only NP is found within the nucleus (Shimuzi et al 1975). With the NP and M monospecific antisera I was able to use indirect immunofluorescence to study measles infected cells. Uninfected cells did not show fluorescence when stained with either the NP and M antisera (Fig. 4c). Infected cells stained with NP antiserum at a 1:20 dilution demonstrated strong fluorescence in the cytoplasm and some fluorescence in the nucleus (Fig. 4a). Infected cells stained with the monospecific antiserum for M-protein at a 1:20 dilution demonstrated fluorescence in the cytoplasm with essentially no fluoresence in the nuclei (Fig. 4b).

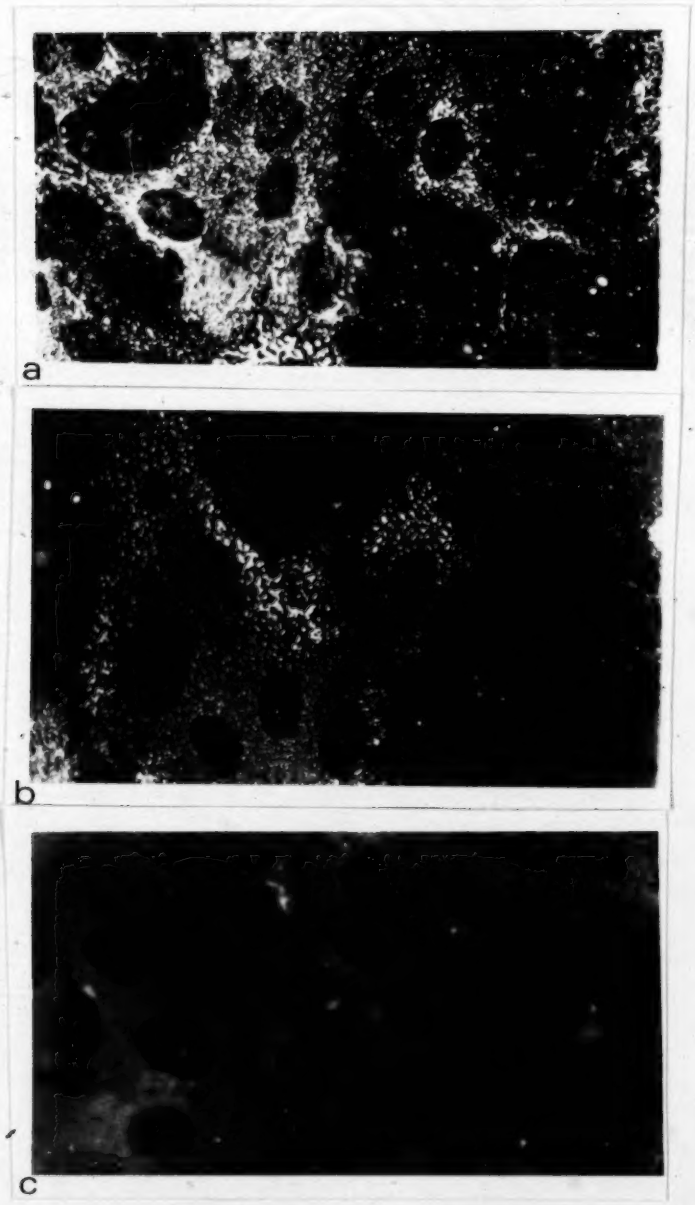

Fig. 4. Immune fluorescence of measles infected Vero cells with the rabbit monospecific antisera to (a) NP protein; (b) M-protein. Uninfected cells are shown in (c).

C) Discussion

The immunoprecipitation technique utilizing the ability of Staphylococcus Protein A to bind to IgG was established to confirm the specificity of antibodies raised in rabbits to the NP and M-proteins of measles virus. The NP and M antisera prepared from denatured antigens were demonstrated to be monospecific. These antisera were used to study the cellular localization of NP and M-proteins in measles infected cells. This study supports the evidence that the NP is present in both the cytoplasm and nucleus of measles infected cells and confirms several other reports in the literature (Wechsler and Fields, 1978; Miller, 1980). Wechsler and Fields (1978a) have reported that L, P, NP and M are present in the nucleus of infected cells. Miller (1980) has reported that these proteins and the HA protein were present in the nuclei of infected cells. I was unable to demonstrate the presence of M-protein in the nucleus, based upon indirect immunofluorescence. In a recent report by Nagura and co-workers (1981), the M-protein could not be demonstrated in the nucleus by electron microscopy (EM) studies using ferritin labelled antibody. They found that the M-protein was primarily present at the sites of viral budding on the plasma membrane and some M-protein in association with cytoplasmic nucleocapsids. In measles infected cells two populations of nucleocapsid have been described, and are referred to as "smooth" nucleocapsids and "fuzzy" nucleocapsids (Fraser and Martin, 1978). "Smooth" nucleocapsids have a diameter of 18 nm, a bouyant density value of ρ 1.323 g/mL and contain virus coded 52S RNA (Kolakofsky et al, 1974). The "fuzzy" nucleocapsids have a bouyant density value of ρ 1.297 g/mL. When visualized by EM, the "fuzzy" nucleocapsid was thought to be formed by an NP-M association (Shimuzu

and Ishida, 1975; Yoshida et al, 1979) and may also include the L and P proteins (Markwell and Fox, 1980). In paramyxovirus-infected cells "fuzzy" nucleocapsid has not been observed in the nucleus (Heggeness et al, 1980). Some of the discrepancies in the literature on the localization of M-protein in the nuclei may be explained by the techniques used. Both Miller (1980) and Wechsler and Fields (1978a) isolated nuclei by detergent extraction and centrifugation. While this technique has long been employed for the rapid isolation of nuclei, it may not be the method of choice. Nuclear fractions isolated by detergent extraction have been shown to contain a significant amount of cytoplasmic material which includes cell organelles (Briedes et al, 1981). This finding and the inability to demonstrate M-protein in the nucleus in our study and others (Nagura et al, 1981; Yoshida et al, 1979), suggests that the presence of M-protein in the nucleus is an artifact as a result of contamination of nuclear fractions with cytoplasmic components.

The appearance of measles virus NP breakdown has been reported previously (Stallcup et al, 1980; Hall et al, 1979). However, the demonstration of intracellular proteolytic cleavage which could be prevented by a protease inhibitor had not been previously shown for measles virus. Purified nucleocapsids of Sendai, NDV and SV-5 have also been shown to be sensitive to proteolysis. Polyacrylamide gel analysis of the nucleocapsids of these viruses after treatment with chymotrypsin or trypsin yields a single protein species of 43 to 45,000 daltons (Mountcastle et al, 1970). This corresponds closely to the molecular weight of the Z protein observed in the immunoprecipitation of measles proteins from infected cells by hyperimmune or NP monospecific serum. This may suggest that the NP of the various paramyxoviruses have large

segments of similar amino acid sequence. The amino acid content of the protease cleaved NP of Sendai virus shows a loss of hydrophobic amino acids (Mountcastle et al, 1974). This may be significant as the M-protein is a hydrophobic protein. The loss of a hydrophobic portion of NP may result in a failure of the M-protein to bind to nucleocapsids.

The nucleocapsids of paramyxoviruses is a flexible helical structure (Compans et al, 1972). It is this flexibility in the nucleocapsid which allows it to be encapsidated into budding particles (Choppin and Compans, 1975). Protease-treated nucleocapsids form rigid rod-like structures which are stable over a wide range of salt concentrations (Mountcastle et al, 1970). This differs from flexible nucleocapsids with uncleaved NP which is flexible at physiological salt concentrations (Heggeness et al, 1980). A rigid nucleocapsid, if not capable of folding, would not be incorporated into budding virus particles (Choppin and Compans, 1975).

The ability of measles virus to establish a persistent infection may require a cell factor (Huang and Baltimore, 1970). It is feasible to consider a persistent infection resulting from proteolytic cleavage of NP. If cleavage of the hydrophobic portion of NP occurs, then failure in the recognition of nucleocapsid by M-protein may result. This would prevent viral budding. An accumulation of nucleocapsids has been observed in both brain tissue from SSPE patients (Katz et al, 1969; Dayan et al, 1967) and in persistently infected cells in vitro (Bernstein et al, 1974; Dubois-Dalcq et al, 1974). A number of cell lines persistently infected with measles virus produce the structural proteins but fail to release "normal" quantities of virus (Wechsler et al, 1979b). Electron microscopic studies of these cells demonstrate an

accumulation of both rigid and flexible nucleocapsids (Oyanagi et al, 1971). In order for this hypothesis to have validity it is essential to correlate the cleavage of NP with a decrease in the release of infectious virus in cell culture systems. Persistently infected cells should demonstrate rapid cleavage of NP, whereas lytically infected cells would not. This to date has not been demonstrated.

The most popular theory for the persistence of measles virus in SSPE brain tissue is a defect in measles virus M-protein. These patients fail to produce antibody to M-protein (Hall et al, 1979). Furthermore, recent studies by Hall and Choppin (1981) have failed to demonstrate M-protein in brain tissue from SSPE patients. Recently, Roux et al (1981) have demonstrated that rapid proteolytic cleavage of M-protein in BHK-cells leads to rapid establishment of persistent infections. It would seem probable that rapid proteolytic cleavage of any structural protein (M or NP in particular) may result in a persistent infection.

CHAPTER IV

Investigation of the Antibody Specificity to Measles Virus Proteins in Immunized Children

A) Introduction

The immunoprecipitation technique was applied to serum samples obtained from children to investigate the antibody specificities in various clinical conditions. Sera from children suffering from atypical measles were analyzed during the acute and convalescent stages of their disease. These children were known to have been immunized with the "killed" measles vaccine. In addition, ten matched serum samples were obtained from children who had received the "killed" vaccine and volunteered for reimmunization with the "live" vaccine. Serum samples prior to and after immunization with "live" measles vaccine (Schwartz vaccine) were obtained by Ms. Margaret King-Collier, a Public Health Nurse with the Edmonton Board of Health and the Division of Infectious Diseases. The serum samples were obtained on a volunteer basis. Parents of the children were informed of the study. The study was also approved by the Edmonton Board of Health and the Edmonton Public School System. The children had no known history of measles virus infection prior to reimmunization and did not exhibit clinical signs of measles following reimmunization.

B) Results

In Fig. 5 the antibody specificity of the acute and convalescent sera from the children who had had atypical measles was compared. As can be seen in Fig. 5c, e, and g, during the acute stage of the disease

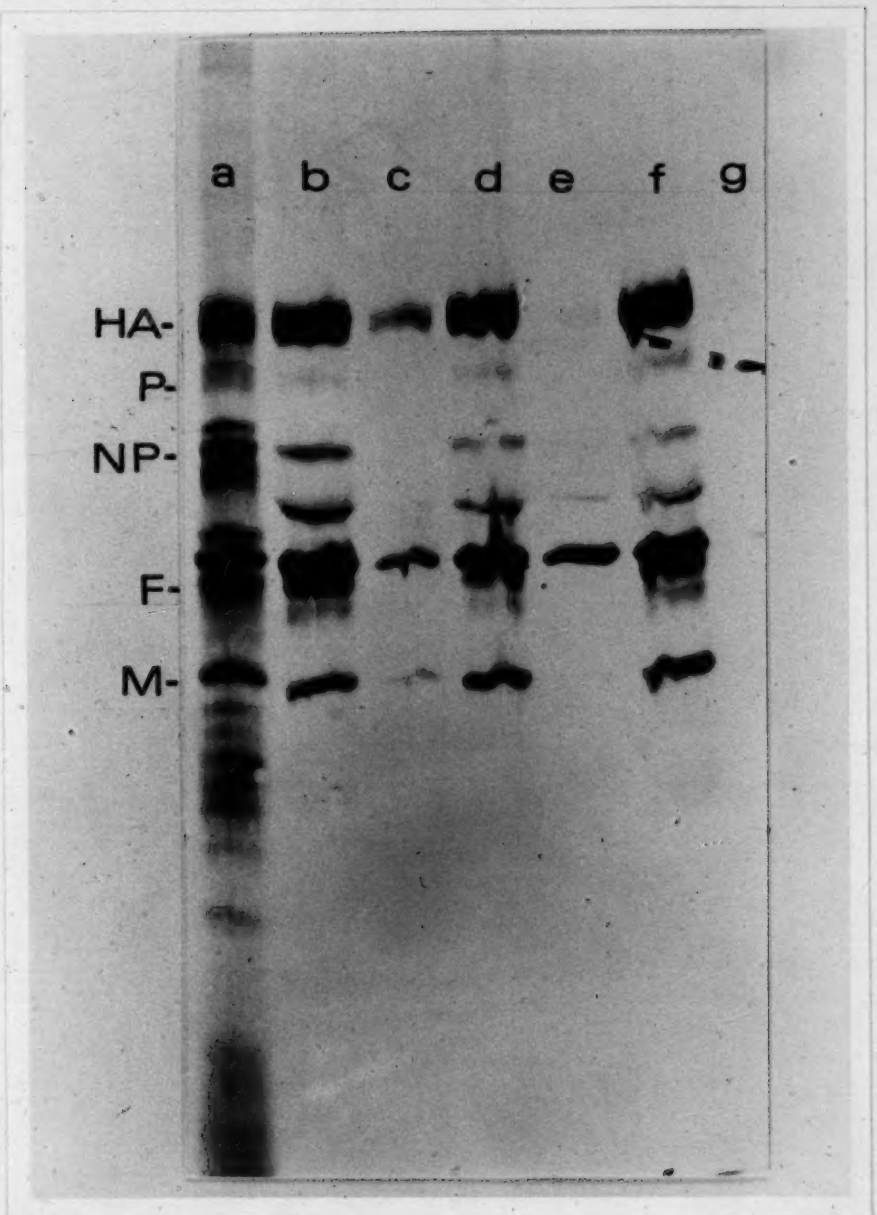

Fig. 5. Autoradiogram of immunoprecipitation of measles virus proteins. Serum from a child immunized with "live" vaccine is shown in a. Matched serum samples from children suffering from atypical measles during the acute stage (c, e, and g) and convalescent stage (b, d and f).

the serum from these individuals contained antibody to the HA, NP and M-proteins. There was no detectable antibody to the F-protein. The convalescent sera collected three weeks following the diagnosis of atypical measles contained antibodies to the HA, NP, F and M-proteins (Fig. 5b, d and f). Fig. 5a shows the immunoprecipitation profile of the serum from a two year old child immunized with "live" vaccine. In this column one can see that the "live" vaccine induces antibody to the HA, NP, F and M-proteins. The "live" vaccine is not known to be followed by atypical measles since there is adequate humoral protection as a result of antibody production to both viral glycoproteins (HA and F).

The protein profile obtained from matched serum samples of the reimmunized children is depicted in Fig. 6. As can be seen, five of the ten children prior to immunization did not have antibodies to F-protein (Fig. 6b, d, g, h and j). Revaccination with the "live" vaccine did not induce the production of antibodies to F-protein in these individuals. Five of the ten children had antibodies to F-protein prior to reimmunization (Fig. 6a, c, e, f and i). This may mean that these individuals had been exposed to measles and developed a relatively mild disease or that the early vaccination program of "killed" vaccine followed by "live" may actually have resulted in adequate immunity in some children. The important point is that revaccination with "live" vaccine did not induce antibody to F-protein in children who did not have antibody to F-protein prior to revaccination.

C) Discussion

The IgG antibody response to the measles virus proteins in matched serum samples from three children with the diagnosis of atypical

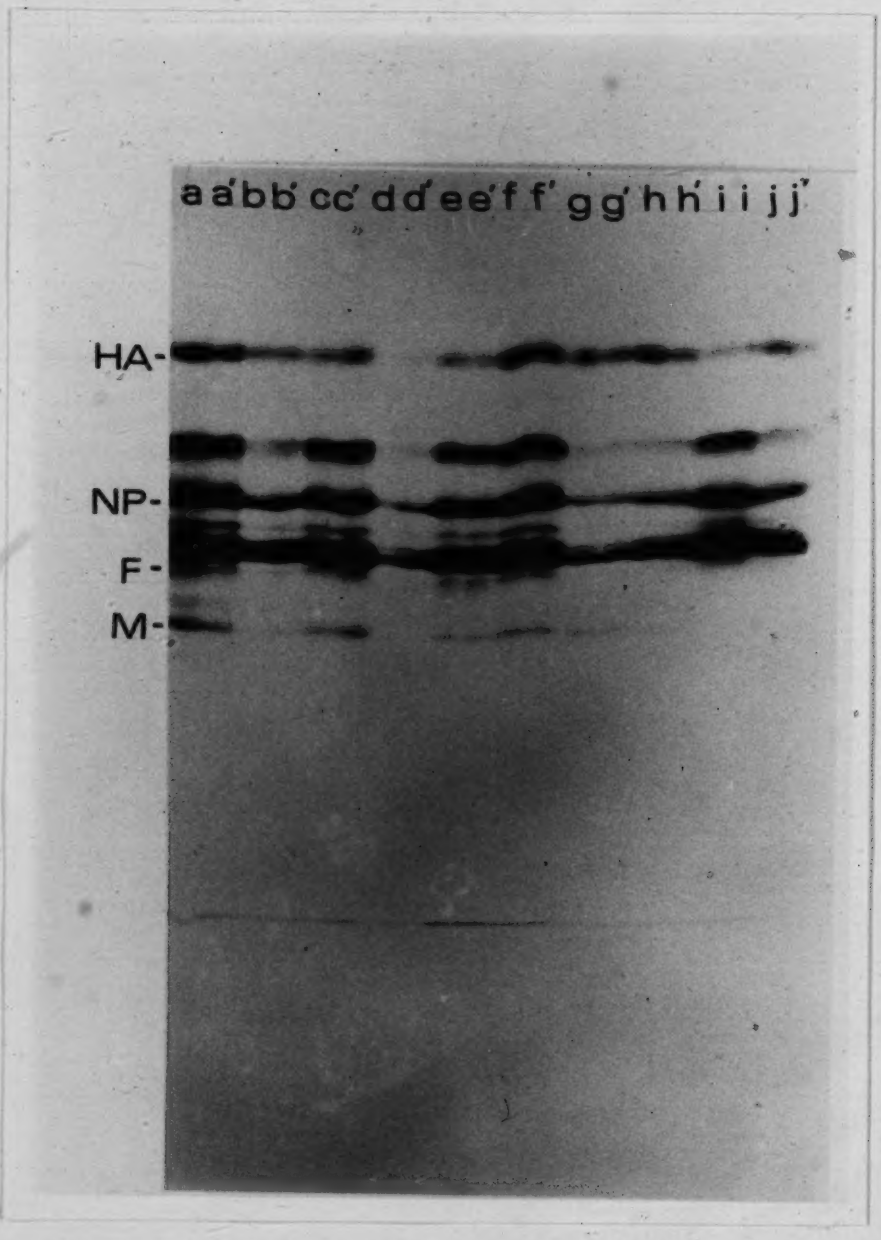

Fig. 6. Autoradiogram of the immunoprecipitated serum samples from children revaccinated with the "live" attenuated measles vaccine. The first letter of each unit, eg. a, denotes preimmune serum; the second letter of each unit, eg. a', denotes post immunization serum.

measles was examined by immunoprecipitation. Serum collected during the acute stage of the disease when a secondary antibody response would be expected to occur precipitated the HA, NP and M-proteins. The convalescent sera, collected three weeks later, precipitated the F-protein of measles virus. The prevailing theory as to the cause of atypical measles is the failure to elicit IgG antibodies to the F-protein following immunization with the "killed" vaccine (Norrby, 1975). This failure has been attributed to the inactivation procedures employed in the preparation of this vaccine, which are believed to destroy the antigenicity of the F-protein (Norrby et al, 1975; Norrby and Penttinen, 1978).

The role of the F-protein has been identified for the paramyxoviruses (Scheid and Choppin, 1974). This protein is responsible for the fusion of the viral membrane to the target cell membrane following attachment of the HA to a cell receptor (Trudgett et al, 1981; Breshkein, 1977). The importance of the F-protein in the spread of the virus infection has been analyzed by Avery and Niven (1979) and Merz et al (1980). These workers have shown that the distal spread of virus infection in cell culture can be inhibited by neutralizing antibodies to the HA-protein. The spread of infection to adjacent cells mediated by cellular membrane fusion was not affected by anti-HA antibodies. Furthermore, the lysis of red blood cells by purified virus was inhibited in the presence of antibodies to the HA-protein. However, hemolysis was not inhibited when virus infected cells are mixed with red blood cells in the presence of HA antibodies. In addition, infection in cell cultures was efficiently neutralized by antibodies to the F-protein. This neutralization was effected on adjacent and distal spread of infection.

These experiments illustrate the importance of antibody to F-protein in the prevention of virus cell to cell spread.

The failure of inactivated measles vaccine to elicit antibodies to the F-protein has lead to the following hypothesis for the induction of atypical measles (Merz et al, 1981): Measles virus infects the cells of the respiratory system with an early dissemination of the virus due to a lag prior to the induction of a secondary antibody response. The infection also spreads to adjacent cells via cell fusion mediated by the F-protein. The viral antigens produced as a result of this early dissemination would in turn serve as a stimulus for a hyperimmune response to the viral proteins (but not to F) since the "killed" vaccine was used for the primary stimulus. With the infected cells providing viral antigens to elicit a secondary antibody response, it has been hypothesized that immune complexes could form in the lungs resulting in complement activation and inflammation. This hypothesis is attractive based on the hyperimmune response to measles antigens in atypical measles. However, there were no immune complexes detected in six children with atypical measles and pulmonary infiltrates (Tyrrell and Dasgupta - personal communication) but, this does not imply that circulating immune complexes contributing to the disease are not present.

After a child has had atypical measles, their serum contains antibodies to F-protein. This might be considered as the "complete" immune response. No child has been recorded as having had a second episode of atypical measles. This has led to the attempt to induce "complete" immunity by revaccination with "live" vaccine after "killed".

The value of revaccination was assessed. My results show that the reimmunization with "live" vaccine of individuals who received the

"killed" vaccine does not induce antibody to the F-protein. In contrast the serum of individuals who received only the "live" vaccine or have suffered an uncomplicated case of measles do exhibit antibodies to F-protein (Fig. 6a; Norrby and Gollmar, 1975; Norrby and Lagercrantz, 1976). The inability of individuals to elicit antibodies to the F-protein following revaccination may in part be due to circulating antibodies to the HA-protein effectively neutralizing the virus (Norrby et al, 1975). This is an attenuated "live" vaccine which may be more susceptible to neutralization than the wild strains.

Following the introduction of a "live" measles vaccine a number of centers in North America conducted "catch up" programs. These programs advocate the use of "live" vaccine to reimmunize individuals who had received the "killed" vaccine (Krugman, 1977b; King-Collier, 1981). However, I was unable to demonstrate the induction of antibodies to the F-protein by this approach in five children, this is in contrast to the report by Norrby and Lagercrantz (1976), and would suggest that the value of revaccination programs is very limited. However, a larger number of serum samples after revaccination should be examined before a final recommendation to discontinue the revaccination program can be given. This study is continuing.

As reimmunization with "live" vaccine may in itself induce atypical measles in some (King-Collier, 1981), it is thought that immunization with purified F-protein should be considered. Immunization with such an immunogen may prevent atypical measles from occurring and would confer "complete" humoral immunity to measles virus in individuals who have received the "killed" vaccine.

In this study, the immunoprecipitation technique was employed to demonstrate that:

a) Individuals immunized with "killed" vaccine do not develop antibodies to F-protein,

b) Individuals who experience atypical measles develop "complete" immunity with antibodies to HA, NP and F-protein,

c) Revaccination with "live" vaccine after "killed" vaccine fails to elicit antibodies to F-protein. This result brings into serious doubt the value of revaccination programs.

CHAPTER V

Production of Monoclonal Antibodies to Measles Virus Protein

A) Introduction

The development of monoclonal antibodies has greatly expanded the ability of scientists to study the molecular biology of viruses. Furthermore, with monoclonal antibodies one has the potential to develop techniques for the purification of specific viral proteins. As was suggested in the previous chapter a purified F-protein would be an ideal immunogen in an attempt to prevent atypical measles in individuals immunized with "killed" vaccine. Another use of monoclonal antibodies would be to compare various strains of viruses. During the recent epidemic of measles in Alberta, properly immunized children were protected to only 70%. Normally this protection should have been in the range of 90-95% (King-Collier, 1981). Antigenic drift has not been described for measles virus. The virus that caused the Alberta epidemic was unusually difficult to isolate (only one isolate between Calgary and Edmonton). These two facts that a) the virus was hard to isolate and, b) the vaccine efficacy was low suggest that antigenic drift might be possible. This would have important implications in a vaccination program with the ultimate aim of measles irradication. This would best be studied with monoclonal antibodies to an isolate obtained by Dr. Tyrrell and myself in 1979, with other wild strains. On this basis, I began a program to produce monoclonal antibodies to measles virus proteins. In this chapter I describe the results of the production of monoclonal antibodies to date. I have not obtained sufficient numbers at this time to compare strains.

B) Results

An indirect RIA was developed to detect monoclonal antibodies specific to the structural proteins of measles virus. The specificity of the RIA was initially confirmed by the following manner. Goat anti-mouse IgG contained no measles antibodies since it failed to immunoprecipitate radiolabelled measles antigens (data not shown). A non-immune mouse serum was used as a negative control. The specificity of the goat anti-mouse IgG was confirmed by the failure of this product to bind to rabbit immune serum used in the RIA. The non-specific binding of radio-iodinated goat anti-mouse IgG was determined by the binding of this serum to cells containing measles antigens but no mouse serum. This is referred to as the background level of the RIA and was approximately 100 CPM. The negative control sera described above did not exceed the background level by more than 100 CPM (see Table 3). The mouse measles immune antisera in my system were examined in five-fold serial dilutions (5^8). Antibodies to measles proteins could be detected in dilutions up to 1:390,625 (Table 3). This corresponds to approximately 0.01 ng of measles specific antibody.

Since hybridoma cells secrete antibodies in µg/ml quantities (Kennett et al, 1980) it was decided that this system was sufficiently sensitive for my purposes of screening hybridoma clones. The fusion of mouse myeloma and spleen cells has been described in Methods and Materials. The hybridoma clones were screened for antibody directed against measles virus proteins 2-3 weeks following the fusion procedure. Each visible colony was screened in triplicate. The negative clones were easily identified as they rarely exceeded the "noise" of the RIA screening system by more than 50 CPM. Putative positive clones were

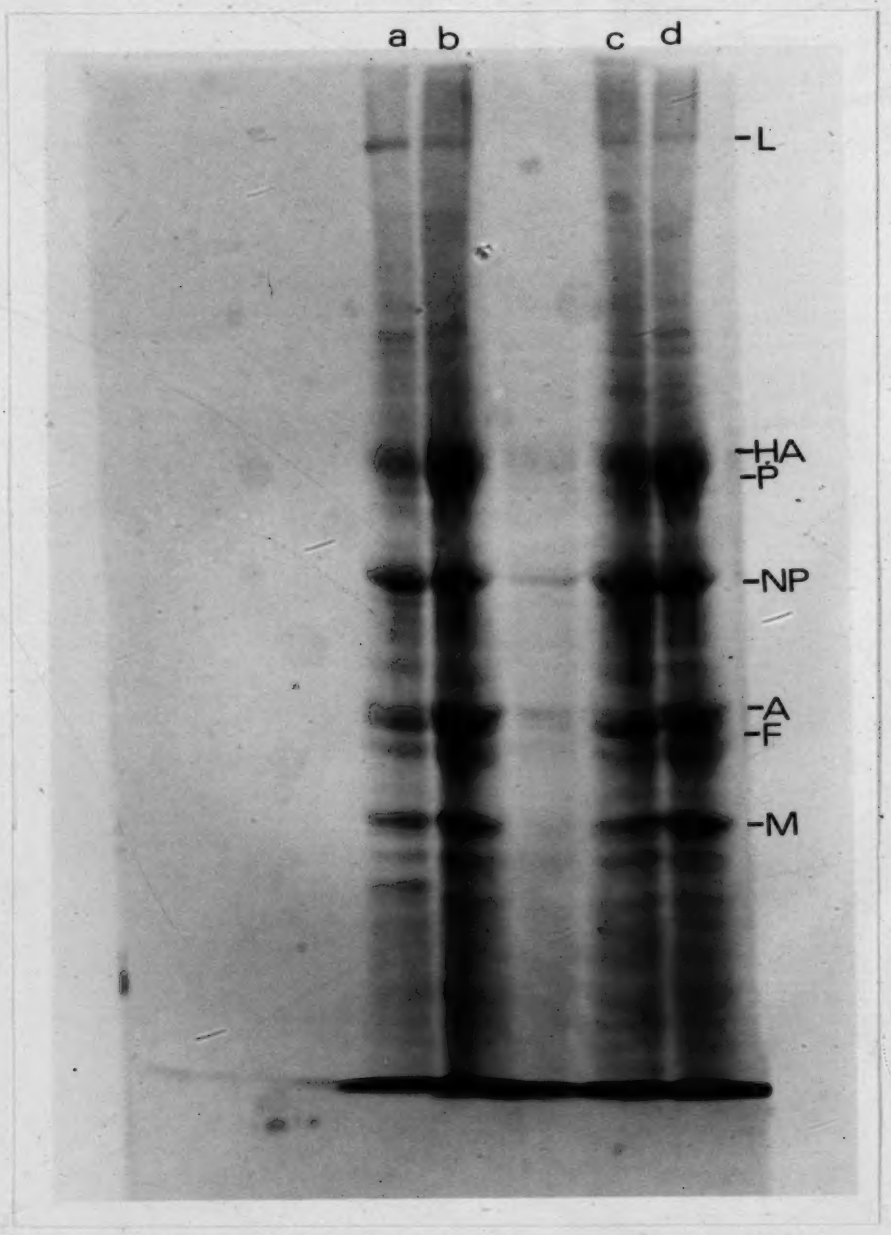

Fig. 7. Coomassie blue stained 10% polyacrylamide gel of the purified measles virus used in the immunization of Balb/c mice. Lanes a-d are the four preparations used.

	Reciprocal serum dilution							
Mouse Immune Serum	5	25	125	625	3,125	15,625	78,125	390,625
1	4,188	3,982	5,994	4,334	2,286	1,124	788	542
2	2,868	3,472	2,896	2,676	1,992	1,668	988	596
3	3,292	2,816	3,040	5,212	2,288	2,124	1,752	776
Mouse Nonimmune Serum								
1	231	248	196	87	210	212	236	197
Rabbit Immune Serum								
1	188	194	204	210	210	185	155	148
BBS - 0.5% BSA	140	148	152	110	137	162	155	148

Table 3. Detection of measles virus antibodies by RIA. Each well was assayed by the addition of 50,000 CPM of ^{125}I-goat anti-mouse IgG. The values are in counts per minute obtained following 10 washes of each well with PBS.

identified on the basis that their CPM were two-fold greater than the negative controls. In most cases these clones were well above this arbitrary cutoff point (Table 4). The positive clones were isolated and propogated in an attempt to establish stable cell lines. In total 17 antibody-secreting clones from six separate experiments were identified. Of these, two clones have been established as stable cell lines, the remaining 15 could not be propagated.

The antibody specificity from positive clones was determined by the immunoprecipitation technique. It was found that the supernatants from positive and negative clones precipitated measles virus NP (Fig. 8d, e and f). This was an unexpected finding which implied that either all the clones screened were specific to measles virus NP, or a component in the culture media was responsible for the precipitation of NP. The first explanation was unlikely. I thought that the fetal calf serum used in the culture media contained maternal IgG antibody to a related paramyxovirus. Cross reactivity between antibodies directed to the NP of measles, rinderpest and canine distemper virus has been documented (Imagawa, 1968; Hall et al, 1980). Immunoprecipitation with 100 µL of RPMI-1640 containing 10% FCS confirmed this hypothesis (Fig. 8c). The apparent discrepancy between the RIA and immunoprecipitation can be easily explained. The fetal calf serum is negative in the RIA since the radiolabelled goat anti-mouse IgG would not detect calf IgG. The immunoprecipitation technique is not species specific since Staphlococcus protein A will bind to IgG from all species.

As antibodies to NP were found in the FCS it was necessary to remove the IgG from serum. This was necessary to clarify interpretation of the specificity of antibody from clones. The bovine IgG molecules

Clone	CPM[a]	Control CPM[b]	Viability
F4P2-B4	388	65	-
F4P4-B3	608	57	-
F4P11-D5	412	89	-
F5P5-A6	438	157	-
F5P5-B6	1,471	157	-
F6P1-B3	417	64	-
F6P1-D4	318	64	-
F6P3-B6	293	31	-
F6P5-A3	371	84	-
F6P6-A1	481	171	-
F6P8-D4	325	77	-
F8P1-A1	725	120	-
F8P1-A3	530	120	-
F9P1-C6	481	88	+
F9P3-B5-A	384	125	+
F9P3-B5-B	175	125	-
F10-P1-A4	310	140	-

a) Average CPM obtained from triplicate wells

b) Average of 10 wells, 5 of which were known negative clones and 5 which serve as background "noise" for each plate

Table 4. Summary table of 17 hybridoma clones secreting antibody to measles virus protein as determined by RIA.

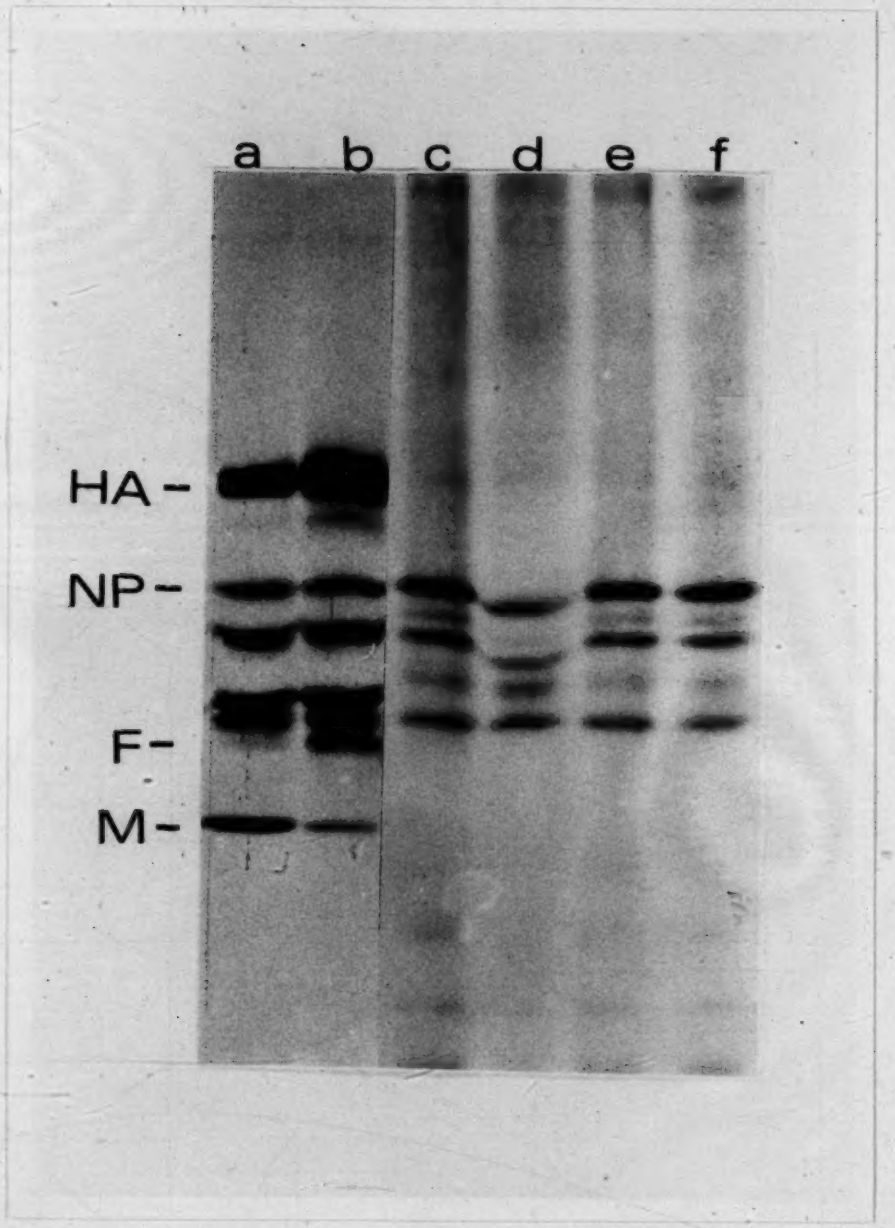

Fig. 8. Autoradiogram of the immunoprecipitation of positive (d and e) clones and a negative clone (f). Lane c is the protein profile obtained with RPMI-1640 10% FCS. Lanes a and b are immunoprecipitates of measles virus by mouse immune serum.

were removed from FCS by the ammonium sulfate precipitation technique (Hudson and Hay, 1976). This serum, following dialysis to remove ammonium sulfate, was found to support growth of clones as well as unprecipitated serum. Immunoprecipitation of 500 µL of media from clone F9P3-C6 (Fig. 9a) and F9P3-B5a (not shown) grown in IgG depleted serum precipitated NP and its breakdown products. The agammaglobulin FCS did not immunoprecipitate measles virus proteins (Fig. 9b).

C) Growth of Clone F9P3-C6 as Ascites Tumors

Inoculation of 1×10^7 cells of clone F9P3-C6 into Balb/c mice primed with pristane (Kennett et al, 1980) and sublethally irradiated (450 rads) (Kennett et al, 1980) induced palable ascites tumors within two weeks in 4 out of 4mice. The ascites fluid was withdrawn by paracentesis. The yield from the four mice was 23.4 ml of monospecific antiserum for NP. Dilutions of the ascites fluid precipitated NP up to a dilution of 1:50 (Fig. 10). The ascites fluid had a total protein content of 9.95 mg/ml (Fig. 11) using the Bio-Rad protein assay system.

D) Discussion

The application of somatic cell hybridization first described by Kohler and Milstein (1975) has been successful for the production of monospecific antibodies to the NP of measles virus. The advantage of using this method for antibody production is that milligram quantities of antibody of monospecificity can be obtained. Thus highly specific probes are available which may allow detailed biochemical studies to be performed. My principal goal utilizing the hybridoma technique was to obtain a series of monoclonal antibodies to the proteins of measles

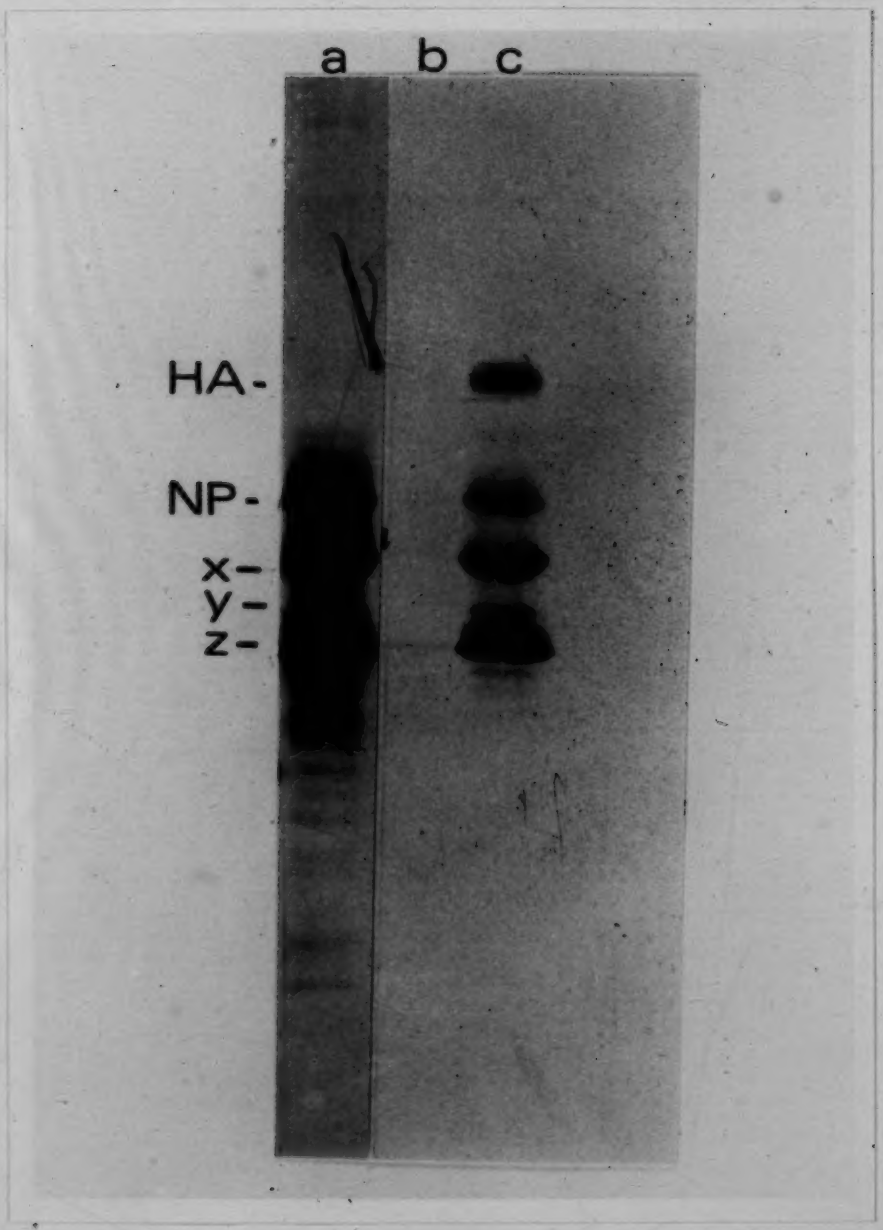

Fig. 9. Autoradiogram of the media, from clone F9-P3-C6 (a) supplemented with ammonium sulfate precipitated serum. Lane b is the immunoprecipitation of the treated FCS and lane c is an immune mouse serum.

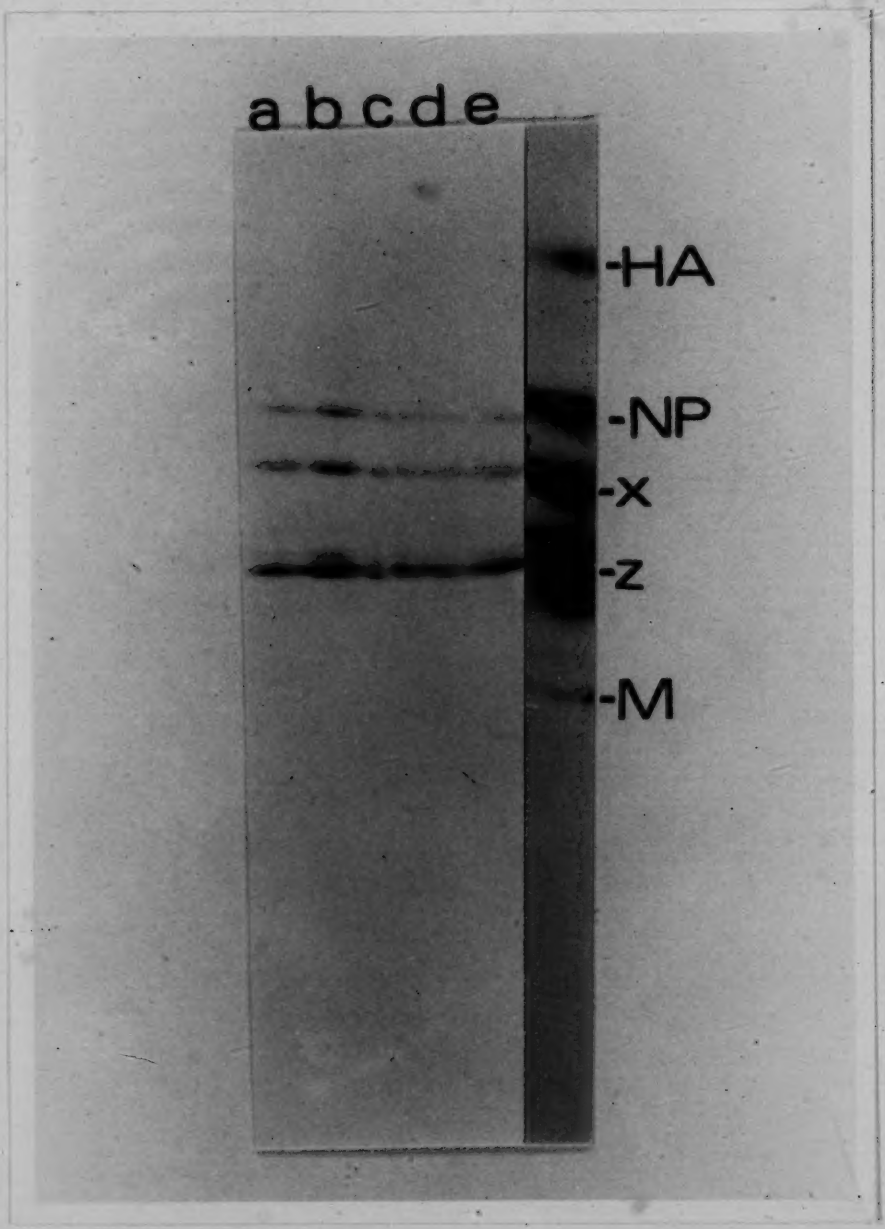

Fig. 10. Autoradiogram of serially diluted ascites fluid containing monospecific antibody to the NP of measles virus from two mice. Lane a is undiluted ascites, lane b 1/5; lane c 1/10; lane d 1/25, and lane e 1/50. The outer lane is a serum control.

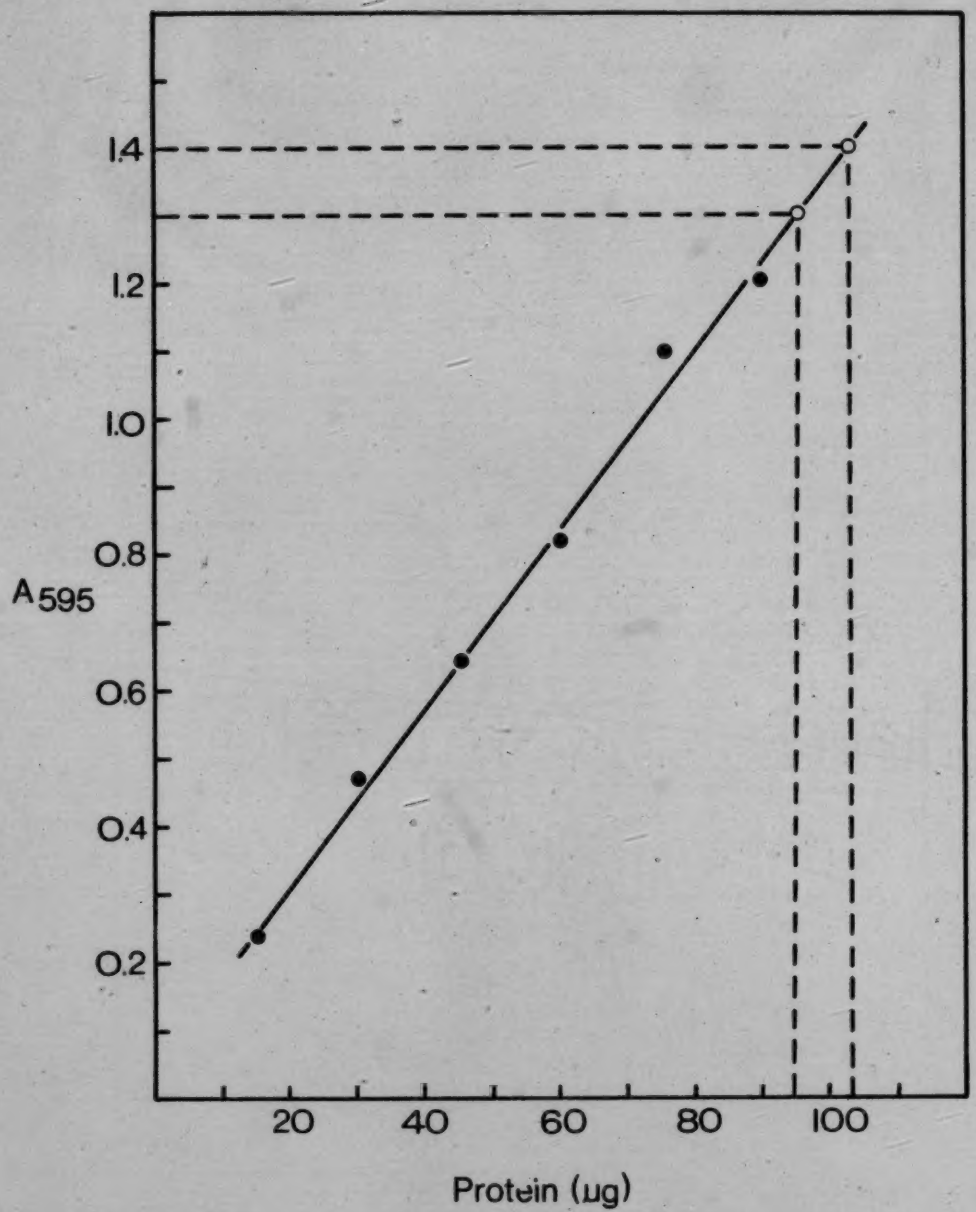

Fig. 11. Protein content of ascites fluid containing monospecific antibody. The total protein content was determined to be 9.95 mg using the BIO-RAD protein assay system. The closed circles denote the protein standard. The open circles denote the values obtained for the ascites fluid containing monospecific antibodies.

viruses. Of 17 positive clones identified I have managed to establish two as stable cell lines.

Other workers have experienced similar problems in maintaining hybridomas whose specificity is directed toward the proteins of measles virus (Birrer et al, 1981). In the case of measles virus, Birrer et al (1981) identified 21 clones secreting antibody specific to the proteins. Of these five clones have been established as stable cell lines. A similar phenomena has been reported for influenza virus (Kaprowski et al 1977). While clone loss is not restricted to these immunogens, there does appear to be an increased incidence of clone loss when these viruses are used as immunogens. The cause of this is unclear. However, I plan to modify the immunization schedules to try to improve the production of monoclonal antibodies to measles. With the experience gained in the production of monoclonal antibodies and development of the RIA systems, I feel that the production of monoclonal antibodies to all the measles antigens will be achieved in the near future.

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